

REMARKS

Claim 1, which is the only claim pending in the application, reads as follows:

1. A congenic rat comprising a mutant GPR10 gene, wherein said congenic rat is obtained by crossing a Otsuka Long-Evans Tokushima Fatty (OTELF) rat (ATCC No. 72016) with a wild-type rat, and wherein said congenic rat exhibits a prolonged immobilization time in a forced swim test compared to said wild-type rat and a prolonged time spent in open arms in an elevated plus-maze test compared to said wild-type rat, and wherein said mutant GPR10 gene contains a G to A substitution at the third position of the coding region.

As described for example at page 1, lines 1-11 of the specification, the claimed congenic rat displays depression and anti-anxiety-like behavior, and is thus useful as a model of depression and anxiety.

Claim Rejections Under 35 USC §§ 101 and 112

At page 2 of the Office Action, the Examiner rejects claim 1 under 35 U.S.C. §101, as lacking patentable utility, and under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The Examiner believes that mutations in GPR10 are manifested as different phenotypes in humans, mice and rats, and thus the claimed congenic rat cannot credibly serve as a model of depression or anxiety. The Examiner bases this conclusion on the following:

- (1) Bhattacharyya et al., *Diabetes* 52:1296-1299 (2003), showing that two particular allelic variants of GPR10 in humans correlate to differences in blood pressure when each is compared to the wild-type allele;
- (2) Gu et al., *J. Neurosci.* 22:93-103 (2003), showing that GPR10 knockout mice exhibit differences in body weight homeostasis as compared to mice having wild-type GPR10; and
- (3) the present specification showing that rats harboring an N-terminal deletion of GPR10 exhibit depression and anti-anxiety-like behavior as compared to rats harboring wild-type GPR10.

The Examiner acknowledges the remarks filed March 10, 2005, in which Applicants explained the following.

The art does not suggest that mutations in GPR10 are correlated to different phenotypes in rat, mouse and human. Genes may be linked to more than one phenotype, and thus finding a correlation between one particular mutation and one particular phenotype does not exclude the possibility that that same mutation, or a different mutation in the gene, could be linked to a second phenotype.

Further, even Bhattacharyya et al., the reference relied upon in the Office Action, show that rats are considered representative of humans with respect to PrRP (the endogenous ligand for GPR10). The Bhattacharyya human study was based upon observations from the rat model. Bhattacharyya thus confirms that GPR10 mutations in both rats and humans can be linked to changes in blood pressure.

Further still, one can only find a correlation between a gene and a particular phenotype, where one actually tests for the particular phenotype. Since Bhattacharyya did not test the human subjects for depression or anxiety, the Bhattacharyya study does not in any way suggest that allelic variants of GPR10 are not also associated with depression or anxiety in humans.

WO 03/080099 actually teaches that human GPR10 is recognized in the art to be associated with diseases of the central nervous system, and that the rat is a recognized model system for studying GPR10.

The Examiner finds these arguments unpersuasive.

The Examiner again asserts that mutations in GPR10 do not manifest the same phenotype in humans, mice and rats, and thus, the claimed congenic rat does not mimic a human condition. The Examiner concludes that the claimed congenic rat does not have patentable utility, and accordingly, has no enabled use.

Applicants Further Response to the Utility and Enablement Rejections

Under the Patent Office's interpretation of section 101, to be patentable, an invention must have at least one utility that is specific, substantial and credible. Screening for compounds useful for treating depression or anxiety is certainly a specific and substantial utility (see MPEP §2107.01).¹ The Examiner's remarks seem to be directed to whether the asserted utility would be viewed as *credible* from the perspective of one of skill in the art.

¹ A utility is "specific" where it is not merely applicable to the broad class of the invention. For example, **screening for compounds useful for treating depression is certainly a specific utility, as it is not broadly applicable to rat experimental biology.**

In fact, the Examiner admits that the asserted utility in the present case is "specific." See page 14, line 8 of the Office Action dated November 12, 2004.

...(footnote continued)

As the Examiner is no doubt aware, a rejection on the basis that an invention lacks a credible utility is a high burden for the Patent Office to meet. The MPEP states that only rarely will an invention have a specific and substantial utility that is not credible. More particularly, The MPEP §2107.02 provides:

Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being “wrong,” even when there may be reason to believe that the assertion is not entirely accurate. Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

As stated in the Amendment filed March 10, 2005, and as further explained herein, Applicants’ asserted utility of using the claimed congenic rat as a model of depression or anxiety *is credible*. Contrary to the Examiner’s contentions, the references cited by the Examiner simply do not suggest in any way that mutations in GPR10 are associated with different phenotypes in mice, rats and humans.

A utility is “substantial” where further research is not needed to identify a “real-world” use for the invention. For example, **screening for compounds useful for treating depression is certainly a substantial utility, because it is a real-world use.**

The Examiner is kindly requested to consider the following additional evidence that Applicants' asserted utility of using the claimed congenic rat as a model for human depression or anxiety is in fact credible.

GPR10 and PrRP are Recognized in the Art as Involved in Blood Pressure, Appetite, Emotional Changes, and Stress Responses in Humans

The attached review article by Ueta et al. (*Exp. Biol. Med.* 228:1168-1174 (2003)) describes the role of so-called feeding-related peptides, including PrRP (the ligand for GPR10), in various physiological roles such as: **blood pressure**, **appetite**, **emotional changes**, and other stress responses (see page 1168). The article further describes a recognition in the art that PrRP is tied to stress responses (see page 1171). The Examiner is also requested to note that this article does not make distinctions about results in different biological systems.

Thus, Ueta shows that **obesity** (alleged by the Examiner to be associated with GPR10 mutations only in mice), **blood pressure** (alleged by the Examiner to be associated with GPR10 mutations only in humans) and **depression/anxiety** (alleged by the Examiner to be associated with GPR10 mutations only in rats) are all believed to be related physiologically through PrRP, and are considered by the art to be in the realm of PrRP's physiological effects in humans through GPR10.

Further, U.S. Patent No. 6,383,764 to Civelli et al. includes "anxiety" as a potential therapeutic use of PrRP, through its interaction with GPR10 (see Table 1).

Thus, the present asserted utility of using the claimed congenic rat harboring a mutation in GPR10 as a model of depression and anxiety is necessarily credible, in view of these documents, and in addition to those cited in the Amendment filed March 10, 2005. Ueta and

Civelli recognize PrRP's potential involvement in human depression or anxiety, and demonstrate that such has been widely proposed in the art. Further, Ueta shows that PrRP is believed to have functions that relate to each of feeding, blood pressure and emotional disorders.

PrRP is Believed in the Art to be Involved in the Regulation of Blood Pressure in Both Rats and Humans

Horiuchi et al., *Brain Research* 958:201-209 (2002), submitted herewith, shows that microinjection into rats of PrRP into the pressor area of the most caudal VLM ("ventrolateral medulla," which is recognized as the caudal pressor area in the rat, elicited dose-dependent increases in **mean arterial pressure**, heart rate, and renal sympathetic nerve activity.

Samson et al., *Brain Research* 858:19-25 (2000), submitted herewith, shows that intracerebroventricular (i.c.v.) administration of both PrRP-20 and PrRP-31 resulted in significantly increased **mean arterial blood pressure** in conscious, unrestrained rats.

Bhattacharyya et al., which shows a correlation between two human variants of GPR10 and blood pressure in humans, correctly observes at page 1296, right column, that PrRP is also known to exhibit effects on blood pressure in the rat.

Thus, the art recognizes that GPR10 plays similar physiological roles in both the human and rat.

Conclusion

In view of the fact that the art supports similar physiological functions for GPR10 in rats and humans, as well as in mice, the use of the claimed congenic rat harboring a mutation in GPR10 as a model of human depression and anxiety, is a specific, substantial and credible

Response under 37 C.F.R. § 1.116
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utility. The Examiner is also reminded of her burden with respect to showing that a specific and substantial utility is not credible.

Accordingly, the specification is also enabling with respect to the use of a rat as a model of human depression and anxiety.

Withdrawal of the rejections are respectfully requested. Reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

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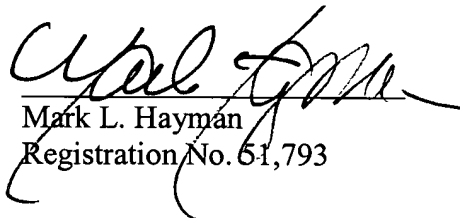
Respectfully submitted,

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON OFFICE

23373

CUSTOMER NUMBER


Mark L. Hayman
Registration No. 61,793

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Involvement of Novel Feeding-Related Peptides in Neuroendocrine Response to Stress

YOICHI UETA,*¹ YUMI OZAKI,* JUN SAITO,* AND TATSUSHI ONAKA†

**Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan, and †Department of Physiology, Jichi Medical School, Tochigi 329-0498, Japan*

Various stressors are known to cause eating disorders. However, it is not known in detail about the neural network and molecular mechanism that are involved in the stress-induced changes of feeding behavior in the central nervous system. Many novel feeding-regulated peptides such as orexins/hypocretins and ghrelin have been discovered since the discovery of leptin derived from adipocytes as a product of the *ob* gene. These novel peptides were identified as endogenous ligands of orphan G protein-coupled receptors. The accumulating evidence reveals that these peptides may be involved in stress responses *via* the central nervous system, as well as feeding behavior. The possible involvement of novel feeding-related peptides in neuroendocrine responses to stress is reviewed here. *Exp Biol Med* 228:1168–1174, 2003

Key words: stress; peptides; G protein-coupled receptor; feeding

Experience has shown that stress is an inducing factor in transient loss of appetite or bulimia and chronic anorexia or overeating. Physiological reactions induced by stress include autonomic nervous responses such as elevated blood pressure, increase of heart rate, or decrease of gastrointestinal motion; endocrine responses such as activation of hypothalamopituitary adrenal (HPA) axis; emotional changes; and behavioral changes such as anorexia or bulimia.

Considerable studies have shown that classic neurotransmitters such as noradrenaline (NA), dopamine, and serotonin and stress hormones (corticotropin-releasing hormone [CRH]) have a contributory role. Recently, many peptides have been termed feeding-related peptides (Table I), and it is currently believed that these peptides, acting as feeding regulatory factors, contribute to various physiological stress reactions (Table II).

G protein-coupled receptors for unknown endogenous ligands are called "orphan receptors." The method for finding endogenous ligand of an orphan receptor is called "reverse pharmacology" because its sequence is reversed from the conventional one in which a physiologically active substance is discovered and its receptor is then identified. Recently a series of novel endogenous ligands have been discovered using this method. Among the feeding-related peptides we discuss, orexins/hypocretins (1), galanin-like peptide (GALP) (2), prolactin-releasing peptide (PrRP) (3), and ghrelin (4) are peptides discovered by this method.

Feeding Regulation by Neuropeptides

Our primitive "appetite" is controlled by a feeding center and a satiety center in the hypothalamus (5). The classic neurotransmitters such as NA, dopamine, and serotonin as well as many neuropeptides play a role in the neural network at these sites. The medial part of the arcuate nucleus (Arc) of the hypothalamus includes numerous neurons that contain neuropeptide Y (NPY), a peptide that powerfully evokes feeding. NPY and agouti-related protein (AgRP) are both present in these neurons. The lateral part of the Arc includes neurons containing pro-opiomelanocortin (POMC) and functions as a feeding inhibition system. The lateral hypothalamic area, known as the feeding center, includes separate neurons containing orexins/hypocretins and melanin-concentrating hormone, respectively, and functions as a feeding stimulation system. The ventromedial nucleus of the hypothalamus is not known to include many neurons containing neuropeptides, but glucose-receptive neurons demonstrate leptin receptors. The hypothalamic paraventricular nucleus (PVN) comprises magnocellular and parvocellular neurons; the magnocellular neurons produce arginine vasopressin and oxytocin (OXT), project their axons into the posterior pituitary, and secrete them into the systemic circulation. The parvocellular neurons in the PVN produce peptides such as CRH and thyrotropin-releasing hormone (TRH) and project their axons into the median eminence, and the peptides they secrete into the hypophyseal portal bloodstream control secretion of anterior pituitary hormones. The parvocellular neurons in the PVN also

¹ To whom requests for reprints should be addressed at Department of Physiology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. E-mail: yoichi@med.uoeh-u.ac.jp

Table I. Feeding Related Peptides

Anorexigenic peptides	Orexigenic peptides
Adrenomedullin	NPY
Bombesin	AgRP
CCK	Galanin
Calcitonin	GHRH
CART	GH
CRH	Ghrelin
Glucagon	MCH
GLP-1	Opioids (β -endorphin)
GRP	Orexin-A
Insulin	-B/hypocretin-1
Leptin	-2
Melanocortin	Prolactin
α MSH	Peptide YY
Neuromedin U	
Neurotensin	
Oxytocin	
PACAP	
POMC	
Somatostatin	
TRH	
Urocortin	
Urocortin II	
Urocortin III	
VIP	

include autonomic neurons with axons projecting to sympathetic preganglionic neurons of the intermediolateral spinal columns.

Feeding Regulation by Peripheral Peptides

After eating, digestive hormones such as cholecystokinin (CCK) transmit neural information regarding satiety to the hypothalamus. This information travels through the abdominal afferent vagus nerves distributed to the stomach and duodenum and is relayed through the nucleus of the

Table II. Contribution of Peptides to Physiological Stress Reactions

	Response to stress	Effect on ACTH secretion
Orexigenic signals		
AgRP	?	Increase
Ghrelin	Activation	Increase
MCH	Activation	Increase
NPY	Activation	Increase
Orexin/hypocretin	Activation	Increase
Anorexigenic signals		
α MSH	Activation	Increase
CART	?	Increase
CCK	?	Increase
CRH	Activation	Increase
Leptin	Suppression or no change	Decrease or increase
Neuromedin U	?	Increase
Oxytocin	Activation	Decrease or increase
PrRP	Activation	Increase
TRH	?	Decrease?

tractus solitarius (NTS) and an intracranial neural circuit. Regulation of OXT secretion by CCK is a well-investigated response.

Leptin is produced by adipocytes and secreted into the systemic circulation. Leptin is believed to act on the satiety center in the hypothalamus to suppress feeding (6). Specifically, it functions as a feeding inhibition system by suppressing the neuronal activity of the Arc containing NPY and triggering accelerated activity of neurons containing POMC. It also promotes energy metabolism by stimulating the sympathetic nervous system.

Ghrelin is a peptide identified recently as an endogenous ligand of growth hormone secretagogue (GHS) analog receptors, and it is produced in large quantities by the stomach (7). Ghrelin in the systemic circulation not only brings about secretion of GH from the anterior pituitary, it also stimulates neuronal activity of the Arc containing NPY/AgRP and otherwise activates the feeding center in the hypothalamus.

Stress Responses and Feeding-Related Peptides

CRH and Urocortin. The center of the physiological endocrine response to stress is the HPA axis. CRH produced in the PVN triggers secretion of ACTH from the anterior pituitary, and ACTH released into the systemic circulation causes secretion of adrenocortical hormones (glucocorticoids) from the adrenal cortex. The fact that central administration of CRH causes various stress-induced stress responses in animal experiments indicates that CRH is a stress hormone in the central nervous system (CNS). Intracerebroventricular administration of CRH in rats also inhibits feeding behavior (8). There are two types of CRH receptors, termed type 1 receptors (CRHR-1) and type 2 receptors (CRHR-2). Endogenous ligands with high affinity for the CRHR-1 and CRHR-2 receptors have been investigated, and peptides identified as demonstrating high affinity to CRHR-2 receptors are urocortin (9), urocortin II (10), (stresscopin-related peptide) (11), and urocortin III (12) (stresscopin) (11). Urocortins II and III demonstrate more selective high affinity to CRHR-2 receptors than urocortin. Recent studies about CRH receptor knockout mice (13, 14) have shown that, in urocortin-induced feeding inhibition, the first half is mediated by CRHR-1 receptors, and the second half is mediated by CRHR-2 receptors. Urocortin II (stresscopin-related peptide) and urocortin III (stresscopin) have also been proven to have a feeding inhibition effect (10, 11).

Orexins/Hypocretins. Orexin-A and -B were discovered as endogenous ligands of G protein-coupled receptors, and hypocretin-1 and -2 were identified through a cDNA library search by a completely separate group. These were identical genetic products (1, 15). Since the discovery of orexins, a great deal of feeding-related research has been carried out on the basis of the localization of orexin-containing neurons in the lateral hypothalamic area and its surrounding area, the lateral hypothalamic area being known as the feeding center. Intracerebroventricular admin-

istration of orexins in the rat and mouse (1, 16) stimulates feeding, and fasting causes increase of orexin mRNA levels (1). It has been also reported that dietary intake is decreased in orexin gene knockout mice (17). However, the effect of intracerebroventricular administration of orexins on feeding is short term and does not change 24-hr total dietary intake or body weight (18, 19). The effect of orexins on feeding is currently attributed to evocation by orexin-induced stimulation of NPY-producing neuron activity in the Arc, where the axons of orexin-producing neurons project (20–25). Many orexin-producing neurons express leptin receptors, and leptin may also regulate the activity of orexin-producing neurons (26, 27).

The parvocellular neurons in the PVN producing CRH have demonstrated abundant expression of the orexin type-2 receptor gene, as well as projection of axons from orexin-producing neurons (28). Intracerebroventricular administration of orexins in the rat has produced the expression of the *c-fos* gene in the parvocellular neurons of the PVN (28–30), secretion of ACTH (29), and elevation of plasma concentration of corticosterone (29–32). The reaction of the secretions of ACTH and corticosterone is completely eliminated by preadministration of the CRH antagonist, α -helical CRH9-41 (32). It is also attenuated by preadministration of NPY antagonists and anti-NPY antibodies (7, 33). Intracerebroventricular administration of orexins in conscious rats increases face-washing behavior, grooming behavior, and exploratory behavior, and these behaviors are inhibited significantly by preadministration of α -helical CRH9-41 (31, 34). Northern blot analysis has demonstrated an increase in the orexin mRNA due to restraint stress and cold stress (31). These results continue to show that the central orexin system is related to physiological stress responses.

Neuromedin U. Neuromedin U (NMU), which is a 23-amino-acid neuropeptide, was discovered from the porcine spinal cord and other tissues (35–37). NMU is widely distributed in the peripheral organs and the CNS (38, 39). Peripheral administration of NMU causes elevation of blood pressure (35), alternation of ion transport (40), and regulation of the adrenocortical function (41). Intracerebroventricular administration of NMU suppresses food intake and heat production (42, 43).

Recent studies have demonstrated that NMU is an endogenous ligand of G protein-coupled receptors, NMU1R, and NMU2R (previously called as FM-3 and FM-4, respectively) (42, 44–45). NMU1R is expressed abundantly in the peripheral organs (42, 44–46), and the expression of NMU2R is mostly restricted to specific regions in the rat brain, in particular the PVN, the wall of the third ventricle in the hypothalamus, and the CA1 region of the hippocampus (42). Ozaki *et al.* demonstrated that intracerebroventricular administration of NMU caused activation of HPA axis and OXT release in rats (47). NMU may be involved in neuroendocrine responses to stress.

Galanin and Galanin-Like Peptide. Galanin is a 29-amino-acid peptide present in the hypothalamus in high

concentrations (48). Intracerebroventricular administration or microinjection of galanin in rats evokes feeding and elevates fat feeding in particular (49). Galanin coexists with arginine vasopressin and CRH in the PVN. Microinjection of galanin in the rat PVN is reported to attenuate ACTH secretion elicited by stress (50). Galanin is known to coexist in the great number of NA-producing neurons in the locus ceruleus, and restraint stress is reported to increase the increase of the expression of the galanin gene (51). Galanin-containing neurons express glucocorticoid receptors, suggesting the relationship with the HPA axis.

GALP was recently identified among galanin receptor subtypes (GALR 1, 2, 3) as an endogenous ligand of GALR2 (2). A recent study demonstrated that dehydration and salt loading markedly increased in the expression of the GALP gene in the pituicytes of rat posterior pituitary (52). Recently, Saito *et al.* demonstrated that the expression of the GALP gene in the posterior pituitary is upregulated during nonosmotic stimuli such as endotoxin shock and chronic inflammatory stress (53).

Oxytocin. Intracerebroventricular administration of OXT inhibits feeding. Treatments intended to promote OXT secretion from the posterior pituitary (e.g., stress, peripheral administration of LiCl and CCK, and gastric distension) also inhibit feeding. CCK is regarded as the physiological satiety signal (54) and the pathway from CCK to OXT secretion is well investigated. First, CCK_A receptors in the stomach are stimulated, the abdominal vagus nerve is activated, NA neurons in the A2 region of the NTS are excited, and NA is released in the hypothalamus, which activates magnacellular OXT neurons (55–57). In addition to the A2 NA neurons (58), NA neurons in the medullary ventrolateral A1 region also play an important role in OXT secretion after stressful stimuli such as noxious stimuli (59). It is unlikely that OXT in the peripheral blood controls feeding directly. At the time when OXT secretion from the posterior pituitary is promoted, OXT release within the hypothalamus has also been shown to be increased, and OXT in the CNS has been shown to inhibit feeding (60). Intracerebroventricular administration of an OXT receptor antagonist attenuates feeding reduction in response to LiCl or CCK (61, 62) and blocks feeding reduction in response to CRH (63). These data suggest that intrinsic OXT may play an important physiological role in inhibition of feeding during satiety and stress. Studies with light microscopy have shown that nerve fibers containing the feeding-inhibition peptides, cocaine-amphetamine-regulated transcript (CART) (64) and PrRP (65), have synaptic contact with hypothalamic OXT neurons. OXT neurons are activated by CART or PrRP administration (66). It has also been suggested that α -melanocyte-stimulating hormone (α -MSH), a feeding inhibition factor released by POMC neurons, activates OXT neurons (67). It is therefore possible that OXT contributes to feeding inhibition by CART, PrRP, and α -MSH. The inhibitory effect of OXT on feeding is blocked by a GLP-1 receptor antagonist. OXT activates GLP-1 neurons in the brain stem. GLP-1

decreases food intake. It is thus possible that GLP-1 is a downstream mediator after activation of OXT neurons (68).

OXT has a short-term feeding inhibition effect. However, when OXT is administered for a long period, it has been reported that food intake becomes increased after an initial decrease in feeding (69). There are no reports that OXT knockout mice get fat. In addition, OXT receptor antagonists also do not block feeding inhibition by α_1 NA receptor agonists (70).

The site of OXT action upon the feeding inhibition is not fully understood. Anatomically, OXT neurons in the PVN project to the medullary dorsal nucleus of the vagus nerve, and microinjection of OXT into the dorsal nucleus of the vagus nerve inhibits gastric motility, suggesting that OXT neurons of the PVN projecting to the medulla oblongata may act to inhibit feeding (60). OXT neurons in the PVN also project to sympathetic preganglionic neurons of intermediolateral nuclei in the spinal columns. OXT has been suggested to excite these neurons. Consequently, it is possible that activation of the sympathetic nervous system is a cause of feeding inhibition. OXT reduces binding affinity in the hypothalamus of α_2 NA receptor agonists that have a feeding promotion effect (71). Thus, modification of NA receptors may also contribute to feeding inhibition by OXT. In addition to feeding inhibition, OXT released in the CNS after various stress stimuli has been proposed to modify neuroendocrine stress responses, such as ACTH secretion, and to affect anxiety behaviors (72).

Prolactin-Releasing Peptide. PrRP was identified as a factor causing release of prolactin from the anterior pituitary *in vitro* (3). PrRP-producing neurons are present in the NTS, the medullary ventrolateral areas, and the hypothalamic ventromedial and dorsomedial areas. The medullary PrRP-producing neurons are A2 or A1 NA neurons producing NA (73). Anatomic data suggest that PrRP fibers have synaptic contact with hypothalamic CRH neurons and OXT neurons (65). Intracerebroventricular administration of PrRP elicits ACTH release and OXT release from the anterior and posterior pituitary (66, 74). Stress activates medullary and hypothalamic PrRP neurons (75, 76). It has been suggested that PrRP and NA may both function cooperatively in neuroendocrine responses to stress (75). Intracerebroventricular administration of anti-PrRP antibodies to rats attenuates OXT secretion in response to conditioned fear (76).

The satiety factor, CCK, activates PrRP neurons. Intracerebroventricular or hypothalamic microinjection of PrRP inhibits feeding (77, 78) but does not induce nausea (conditioned taste aversion) (79). PrRP also increases body temperature. From these data, PrRP has been proposed to mediate satiety signaling (79). PrRP neurons express leptin receptors and PrRP mRNA is reduced when leptin signaling is defective (e.g., fasting, Zucker rats) (80). Thus PrRP is regulated by leptin. PrRP promotes release of the feeding inhibition factors, α -MSH and neurotensin (78). It is thus possible that α -MSH and neurotensin contribute to the in-

hibitory effect of PrRP. The action of PrRP on feeding inhibition during stress requires further investigation.

Leptin. Leptin synthesized by adipocytes acts on the brain to inhibit feeding (6). The targets of leptin in the brain are believed to be the Arc, the hypothalamic ventromedial nucleus, and the hypothalamic dorsomedial nucleus. Leptin receptors are present in these regions. For example, leptin receptors (OB-Rb) are present in NPY neurons of the Arc, which have a feeding promotion effect, and in POMC neurons of the Arc, which have a feeding inhibition effect. Leptin inhibits NPY neurons and activates POMC neurons. When leptin signaling is deficient, hypothalamic NPY and AgRP mRNA levels are increased, and POMC and CART mRNA levels are decreased. Leptin also raises energy consumption. This is partly due to the fact that leptin activates hypothalamic neurons projecting to sympathetic preganglionic neurons of intermediolateral nuclei in the spinal columns.

Leptin concentration in the blood decreases during hunger. In the state of hunger, the HPA system is activated, whereas thyroid, reproductive, and growth hormone systems are suppressed. Body temperature and energy metabolism decline. At such time, immune function is depressed. These responses are attributed to physiological adaptive phenomena responding to hunger (6). Decrease in leptin concentration may contribute to these responses. The HPA system is the primary system responding to stress, and the effect of leptin on the HPA system is deemed inhibitory. Intracerebroventricular administration of leptin attenuates stress-induced responses in hypothalamic CRH mRNA increase and ACTH release from the pituitary. Leptin has been suggested to inhibit activity of NPY neurons and NA release within the PVN (81), and NPY and NA have been shown to activate CRH neurons. Leptin may also reinforce glucocorticoid feedback inhibition by increasing the expression of PVN and hippocampal glucocorticoid receptors (82). It has also been suggested that leptin acts on adrenal cortex levels and inhibits glucocorticoid release (83, 84). On the contrary, some studies report that leptin activates the HPA axis (85, 86). Meanwhile, leptin production is stimulated by glucocorticoids, insulin, adrenocortical hormones, estrogen, and cytokines, whereas it is inhibited by adrenalin and testosterone.

Ghrelin. Ghrelin was discovered as an intrinsic ligand of GHS receptors (GHS-R) (4). Ghrelin is produced primarily in the stomach, but production in the colon, pituitary, kidneys, placenta, and hypothalamus has also been discovered. Ghrelin acts on the pituitary or the hypothalamus to bring about release of GHRH or an unknown factor (U-factor) and thereby promote GH secretion from the pituitary (87). Ghrelin also evokes a hunger sensation in the human and promotes feeding, perhaps *via* activation of GHS-R in a separate system. The blood concentration of ghrelin is decreased by satiation or feeding and is conversely increased during fasting or hunger or in anorexia nervosa. There is evidence that ghrelin acts on the hypothalamus to promote feeding. Microinjection of ghrelin to the Arc, the PVN, the

hypothalamic dorsomedial nucleus, and the hypothalamic ventromedial nucleus promotes feeding (88). Ghrelin neurons have been shown to lie in the space between the lateral hypothalamus, arcuate, ventromedial, dorsomedial, and paraventricular hypothalamic nuclei (89). Ghrelin administration activates NPY/AgRP neurons in the Arc (90), and the feeding promotion effect of ghrelin has been shown to be blocked by NPY Y1 receptor antagonists, antibodies to NPY, antibodies to AgRP, and antibodies to orexin (91); it is therefore possible that the feeding-promotive effect of ghrelin is mediated by NPY/AgRP neurons in the Arc and orexin neurons in the lateral hypothalamic area. However, the action of ghrelin has also been observed in NPY knock-out mice. It also has been suggested that ghrelin stimulates the hypothalamic feeding center *via* activation of the vagus nerve (92).

Ghrelin receptors are distributed widely within the brain: the hypothalamus, hippocampus, substantia nigra, ventral tegmental area, and raphe nuclei. Ghrelin may therefore have physiological functions other than feeding. Ghrelin has the opposite effect of leptin in metabolism. Ghrelin decreases oxidation of fats and causes fat accumulation. The function of ghrelin during stress remains to be determined. A recent report states that ghrelin augments ACTH release in response to stress secretion-inhibiting effect of leptin (81). In this respect, it is interesting to point out that ghrelin reduces γ -aminobutyric acid release presynaptically in the PVN (89).

Conclusion

We summarize the topic of stress and feeding-related peptides with emphasis on recently discovered novel peptides. Future understanding of the contribution of feeding-related peptides to physiological stress responses holds the key to prevention and treatment of various stress-induced illnesses.

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Research report

Effects of prolactin-releasing peptide microinjection into the ventrolateral medulla on arterial pressure and sympathetic activity in rats

Jouji Horiuchi^a, Takeshi Saigusa^b, Nobuhiro Sugiyama^c, Shigenobu Kanba^c,
Yasuhiro Nishida^a, Yu Sato^b, Shuji Hinuma^d, Jun Arita^{b,*}

^aDepartment of Physiology, The National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

^bDepartment of Physiology, Yamanashi Medical University, 1110 Shimokato, Tamaho, Yamanashi 409-3898, Japan

^cDepartment of Psychiatry, Yamanashi Medical University, Tamaho, Yamanashi 409-3898, Japan

^dPharmaceutical Discovery Research Division, Takeda Chemical Industries, Tsukuba, Ibaraki 300-4293, Japan

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Abstract

Prolactin-releasing peptide (PrRP), originally isolated from the hypothalamus, is highly localized in the cardiovascular regions of the medulla, and intracerebroventricular administration of PrRP causes a pressor response. In the present study we investigated the cardiovascular effects of PrRP applied to functionally different areas of the ventrolateral medulla (VLM), and to the nucleus tractus solitarius (NTS) and the area postrema (AP). In urethane-anesthetized rats, microinjection of PrRP into the pressor area of the most caudal VLM, recognized as the caudal pressor area in the rat, elicited dose-dependent increases in mean arterial pressure, heart rate, and renal sympathetic nerve activity. In the same injection area, neither thyrotropin-releasing hormone, corticotropin-releasing hormone nor angiotensin II affected these baseline cardiovascular variables. On the other hand, microinjection of PrRP into more rostral parts of the VLM, i.e. the depressor area of the caudal VLM and the pressor area of the rostral VLM, as well as the NTS and the AP, had no effect on these cardiovascular variables. Immunohistochemical analysis in the medulla revealed that the cardiovascularly PrRP-responsive region contained PrRP-immunoreactive cell bodies and nerve fibers. These results suggest that the most caudal VLM is an action site of PrRP to induce a pressor response, which is mediated, at least partly, by the increase in sympathetic outflow.

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1. Introduction

A neuropeptide that is able to stimulate prolactin release from anterior pituitary cells was recently isolated from the hypothalamus as a ligand to an orphan receptor, and named prolactin-releasing peptide (PrRP) [11]. Interestingly, neurons expressing PrRP or PrRP mRNA are distributed not only in the hypothalamus but also in discrete regions

within the medulla, including the ventrolateral medulla (VLM) and the nucleus tractus solitarius (NTS) [5,18,20,22,28,33]. PrRP-receptor mRNA was also detected in the medulla, abundantly in the area postrema (AP) and the NTS, and less in the reticular formation including the VLM [28]. These morphological findings have raised the possibility that the peptide participates in a wide spectrum of autonomic functions in which brainstem neural structures are involved.

The VLM and the NTS contain the cardiovascular neurons that play essential roles in the tonic and phasic

*Corresponding author. Fax: +81-55-273-6730.

E-mail address: jarita@res.yamanashi-med.ac.jp (J. Arita).

control of sympathetic nerve activity (SNA) and thereby blood pressure (See [6,10,31] for reviews), whereas the AP modifies cardiovascular function as a circumventricular organ in the brain stem [2]. On the basis of its functional and anatomical natures, the VLM is further divided into the following three regions. First, the rostral VLM, a pressor area located rostral to the lateral reticular nucleus, contains a group of sympathoexcitatory neurons that project to sympathetic preganglionic neurons in the spinal cord [23] and provides a major source of SNA. Secondly, the caudal VLM, a depressor area located around the level of the obex, contains tonically active sympathoinhibitory neurons projecting to the rostral VLM [3]. These cardiovascular neurons in the rostral and caudal VLM, as well as those in the NTS—the main site of termination of cardiovascular primary afferent fibers [31]—constitute a core circuit responsible for the sympathetic baroreflex and other reflex cardiovascular responses. Thirdly, another pressor area has been identified in the caudal end of the VLM, located more caudally to the depressor area, and therefore recognized as the caudal pressor area (CPA) [4,24,26]. Although the neurons in the CPA exert a tonic excitatory effect on the sympathetic premotor neurons in the rostral VLM [4], little is known about the physiological and functional role of the CPA in cardiovascular regulation.

These medullary regions contain a variety of neuropeptides and catecholamines, which affect the core circuit responsible for cardiovascular regulation as neurotransmitters or neuromodulators [2,8,10,12,27]. In the caudal regions of the VLM and in the NTS, noradrenaline-containing neurons are localized in clusters [14] and are termed the A1 and A2 cell groups, respectively. The A1 and A2 neurons are thought to participate in the neural and humoral cardiovascular responses induced by stress and other stimuli via their ascending projections to the hypothalamus [1,9]. Interestingly, PrRP-immunoreactive (PrRP-ir) neurons localized in the caudal VLM and NTS also exhibit tyrosine hydroxylase (TH) immunoreactivity and therefore are assumed to be a subset of the A1 and A2 neurons, respectively [5,20,22,28]. Taken together, it is very likely that the PrRP-containing neurons in the medulla may participate in cardiovascular regulation. Supporting this possibility, Samson et al. [29] have demonstrated that intracerebroventricular (i.c.v.) administration of PrRP causes an increase in arterial pressure in conscious rats.

Although involvement of the caudal medulla has been proposed, the precise action sites of PrRP and its pressor mechanism have not yet been identified. Therefore, in the present study, we investigated the effects of unilateral PrRP microinjection into the functionally different cardiovascular regions within the VLM, as well as into the NTS and the AP, on mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (rSNA). Furthermore, we investigated the precise neuroanatomical relationship between PrRP-ir neuronal components and the cardiovascularly PrRP-responsive regions in the medulla.

2. Materials and methods

2.1. Microinjection and cardiovascular recordings

Experiments were performed on male Sprague–Dawley rats (10–14 weeks, Clea Japan, Tokyo, Japan) weighing 350–550 g. All experiments were carried out in accordance with the guidelines for animal experimentation of the Japan Physiological Society. Rats were anesthetized with urethane (1.3–1.4 g/kg, i.p.). Surgery, microinjection, and recordings were performed as described previously [32]. Body temperature was maintained in the range of 37–38 °C with a water-circulating heating pad. The trachea was cannulated and catheters were placed in the femoral artery and femoral vein for the recording of pulsatile arterial pressure and drug application, respectively. MAP and HR were derived from the pulsatile signal of arterial pressure by means of a low pass filter and a rate meter, respectively. The renal sympathetic nerve on the left side was isolated from surrounding connective tissues and its activity was recorded using a bipolar stainless-steel electrode. The signal from the electrodes was amplified, passed through a band pass filter (50–1000 Hz), and then rectified and integrated (resetting every 1 s). After completion of all surgical procedures, neuromuscular blockade was induced by gallamine triphenylchloride (0.2 mg/kg i.v. every 1–2 h), and the animals were artificially ventilated with a respiratory pump at a level that maintained end-tidal CO₂ close to 4%. The effects of gallamine triphenylchloride were allowed to wear off before each additional dose was administered. The adequacy of anesthesia during neuromuscular blockade was verified by stable baseline arterial pressure, HR, and renal SNA (rSNA).

The rats were fixed on a stereotaxic apparatus (ST-7, Narishige, Tokyo, Japan) in a position where the incisor bar was lowered maximally (17 mm below horizontal zero), and the dorsal surface of the medulla was exposed. Microinjection was performed unilaterally using a micropipette held in a micromanipulator tilted at an angle of 20° rostral. The rostrocaudal, mediolateral, and dorsoventral coordinates of the injection sites were determined with reference to obex, the midline, and the dorsal surface of the medulla, respectively. Injections were made by pressure, and the volume injected, 50 nl, was measured by the displacement of the meniscus in the pipette with reference to a horizontal grid viewed through an operating microscope. The compounds injected were 50 mM sodium glutamate (Wako Chemical, Osaka, Japan), 0.015, 0.15 or 1.5 mM PrRP (Takeda Chemical or Peptide Institute, Osaka, Japan), 1 mM angiotensin II (Sigma Chemical, St Louis, MO, USA), 1 mM thyrotropin-releasing hormone (TRH, Peptide Institute) or 1 mM corticotropin-releasing hormone (CRH, Peptide Institute). The vehicle solution was artificial cerebrospinal fluid (144 mM NaCl, 1.2 mM CaCl₂, 2.8 mM KCl, and 0.9 mM MgCl₂) adjusted to pH 7.4.

Three different cardiovascular regions in the VLM were identified as the sites at which microinjection of sodium glutamate (30–50 nl of 50 mM solution) evoked a pressor or a depressor response. The pressor area within the rostral VLM was identified as the site at which glutamate microinjection evoked a pressor response of at least 20 mmHg and an increase in rSNA of at least 40% with respect to the pre-injection level. The depressor area in the caudal VLM was identified as the site at which glutamate microinjection evoked a depressor response of at least 20 mmHg and a decrease in rSNA of at least 30% with respect to the pre-injection level. Similarly, the pressor area within the caudal VLM was identified as the site at which glutamate microinjection evoked a pressor response of at least 20 mmHg and an increase in rSNA of at least 30% with respect to the pre-injection level. When microinjections of different doses of PrRP were made into the caudal pressor VLM region, there was a waiting period of at least 30 min between the microinjections. The cardiovascular region in the medial part of the NTS (0.5 mm rostral to the obex, 0.6 mm lateral to the midline, and 1.5 mm ventral to the dorsal surface) was identified similarly to the VLM. This was by means of a glutamate microinjection that evoked a depressor response of at least 30 mmHg and a decrease in rSNA of at least 50% with respect to the pre-injection level. In contrast to these injection sites, the AP was identified visually under an operation microscope. The microinjections of glutamate and PrRP were made into the midpoint between the rostral edge and the caudal end of the AP on the midline and 0.3–0.4 mm deep from the dorsal surface of the AP.

At the end of each experiment, the injection site in the caudal VLM was marked by 50 nl of 1% Pontamine sky-blue. Rats were then perfused transcardially with 500 ml of heparinized saline followed by 500 ml of 4% paraformaldehyde solution. Frozen coronal sections (50 μ m) of the medulla were cut and stained with 0.5% cresyl violet to identify the injection site under a microscope.

Comparisons between the responses evoked by microinjection of the vehicle solution and the different doses of PrRP were made using one-way analysis of variance. Comparisons between responses evoked by the microinjection of a particular dose and that of the vehicle solution were made using the paired *t*-test with Bonferroni adjustment for multiple comparisons. A *P* value of less than 0.05 was regarded as statistically significant. All values are indicated as mean \pm S.E.M.

2.2. Immunostaining for PrRP in the medulla

Rats were anesthetized with 50 mg/kg sodium pentobarbital and perfused through the aorta at a flow rate of 20 ml/min with 30 ml of normal saline containing 2% sodium nitrite, followed by 200 ml of 4% paraformaldehyde containing 2% acrolein (Merck, Darmstadt, Germany) in 0.1 M sodium phosphate buffer, pH 7.2. After

fixation, the rats were again infused with saline to flush the residual acrolein from the vasculature. Brains were then removed, immersed overnight in 0.1 M phosphate buffer containing 20% sucrose, and frozen using dry ice. A total of 120 serial coronal sections (40 μ m) were obtained from individual frozen brains, extending from the rostrocaudal level of the most rostral part of the cervical cord (C1) to that of the middle part of the fourth ventricle in the medulla. They were collected into a cryoprotectant (25% ethylene glycol and 25% glycerine in 0.05 M phosphate buffer) and then stored at -20°C before immunohistochemical processing to detect PrRP.

The sections were divided into the following three groups: (1) for Nissl staining using cresyl violet; (2) for PrRP staining using P2L-1T, a mouse monoclonal antibody that recognizes PrRP present in both cell bodies and nerve fibers; and (3) for PrRP staining using P2L-1C, an antibody that recognizes PrRP only in nerve fibers [18]. Free-floating sections to be immunostained for PrRP were treated with 1% sodium borohydride solution for 20 min. After extensive rinsing, the sections were quenched with 1% hydrogen peroxide for 30 min and blocked with 10% normal horse serum for 30 min. Thereafter, sections were incubated with either one of the monoclonal antibodies against PrRP at appropriate dilutions in Tris-buffered saline (TBS) containing 10% horse serum at 4°C . After 48 h of incubation, the sections were rinsed three times in TBS containing 0.3% Tween-20 and incubated for 60 min with biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) at a 1:200 dilution in TBS containing 10% horse serum. The sections were rinsed and incubated with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector) in TBS for 60 min. The peroxidase-substrate reaction was performed for 5 min using the DAB Peroxidase Substrate Tablet Set (Sigma Chemicals) in the presence of 1% nickel ammonium sulfate. The sections were mounted on gelatin-coated glass slides, air-dried, dehydrated with ethanol, cleared with xylene, and coverslipped with Histomount (Zymed, San Francisco, CA, USA). Neural structures in the medulla were identified according to the rat brain atlas by Paxinos and Watson [25].

3. Results

3.1. Cardiovascular responses induced by microinjection of PrRP into three different rostrocaudal levels of the VLM, and into the NTS and AP

In six barointact rats, microinjection of PrRP (75 pmol) was made into a total of 142 histologically identified sites in the rostral and caudal VLM (Fig. 1). Preinjection levels of MAP and HR were 95 ± 3 mmHg and 359 ± 9 bpm, respectively. Significant increases in MAP (>10 mmHg), HR (>20 bpm), and rSNA ($>15\%$ of the pre-injection

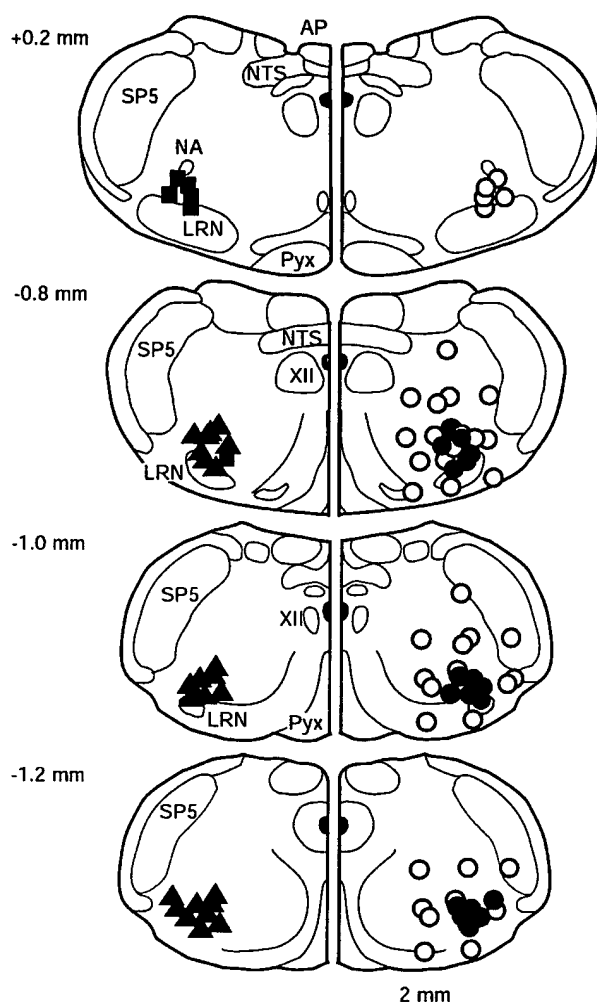


Fig. 1. Distribution of the sites of PrRP microinjection on coronal sections through the caudal part of the medulla oblongata. The sites of sympathoexcitatory-pressor response (closed circles) obtained by PrRP (75 pmol in 50 nl) are shown in the right panels, regardless of the side on which the injections were actually made. The sites of no response or slight responses to injection of PrRP are shown with open circles. The sites of sympathoinhibitory-depressor response (closed squares) or sympathoexcitatory-pressor response (closed triangles), evoked by glutamate injections (2.5 nmol in 50 nl), are shown in the left panels. The distance (in mm) of each section from the obex is indicated in the upper left corner. AP, area postrema; NTS, nucleus of the solitary tract; SP5, spinal trigeminal nucleus; NA, nucleus ambiguus; LRN, lateral reticular nucleus; Pyx, pyramidal decussation; XII, hypoglossal nucleus.

level) were elicited when PrRP was injected into the sites within a highly restricted region. This region corresponded to the following stereotaxic coordinates: 0.8–1.3 mm caudal to obex, 1.3–2.2 mm lateral to the midline, and 1.4–2.2 mm from the dorsal surface of the medulla (Figs. 1 and 2). The sites where PrRP induced significant increases in these cardiovascular variables overlapped well with the pressor area in the most caudal VLM, which was identified by microinjections of glutamate (2.5 nmol, Figs.

1 and 2). Typically the MAP and rSNA began to increase within 3 s after the injection, reached a peak at approximately 2 min (Fig. 2), and remained elevated for 5–15 min. Microinjections of PrRP outside this pressor region at the same rostrocaudal levels elicited very little or no changes in MAP, HR, and rSNA (Fig. 1). In contrast, microinjection of PrRP did not elicit any significant changes in MAP, HR, and rSNA either in the pressor area of the rostral VLM or in the depressor area of the caudal VLM, where microinjection of glutamate (1.5–2.5 nmol) caused profound increases or decreases in MAP, HR, and rSNA, respectively (Fig. 2).

In six rats, microinjection of three different doses of PrRP into the most caudal VLM produced dose-dependent increases in MAP, HR, and rSNA (Fig. 3). Microinjection of the lowest dose (0.75 pmol) caused slight increases in MAP, HR, and rSNA (2 ± 3 mmHg, 3 ± 3 bpm, and $3 \pm 2\%$, respectively), but these changes were not significantly different from those induced by the same amount (50 nl) of vehicle solution. In contrast, microinjection of higher doses (7.5 and 75 pmol) of PrRP elicited significant increases in MAP, HR, and rSNA. The differences from control levels in MAP, HR, and rSNA that were elicited by 75 pmol PrRP were 18 ± 3 mmHg, 13 ± 3 bpm, and $40 \pm 6\%$, respectively.

In four animals, 50 pmol of either angiotensin II, CRH or TRH was injected into the pressor site in the most caudal VLM to validate the specificity for the PrRP action. There were no detectable changes in the resting levels of MAP, HR, and rSNA after these peptides were injected whereas 2.5 nmol of glutamate successfully produced pressor and sympathoexcitatory responses (Table 1).

In the same animals, microinjections of PrRP (75 pmol) were made into the NTS cardiovascular region. Microinjection of glutamate (2.5 nmol) in the medial part of the NTS caused profound decreases in MAP, HR, and rSNA, but microinjection of PrRP into the same site of the NTS did not elicit any significant changes in the resting levels of MAP, HR, and rSNA (Table 2). In the same animals, neither glutamate nor PrRP injected into the AP induced any detectable changes in the resting levels of MAP, HR, and rSNA (Table 2).

3.2. The distribution of PrRP-immunoreactive cell bodies and nerve fibers in the medulla

Immunohistochemical studies were performed to examine the precise neuroanatomical relationship between the PrRP-responsive cardiovascular region and PrRP-containing neurons in the medulla. PrRP-ir cell bodies, which were detected only by the P2L-1T antibody, were clustered exclusively in two regions of the caudal medulla, i.e. the most caudal VLM and the NTS. In the most caudal VLM, small numbers of PrRP-ir cell bodies were scattered in the caudal part of the caudoventrolateral reticular nucleus (Fig. 4A). These cell bodies extended rostrocaudally from the

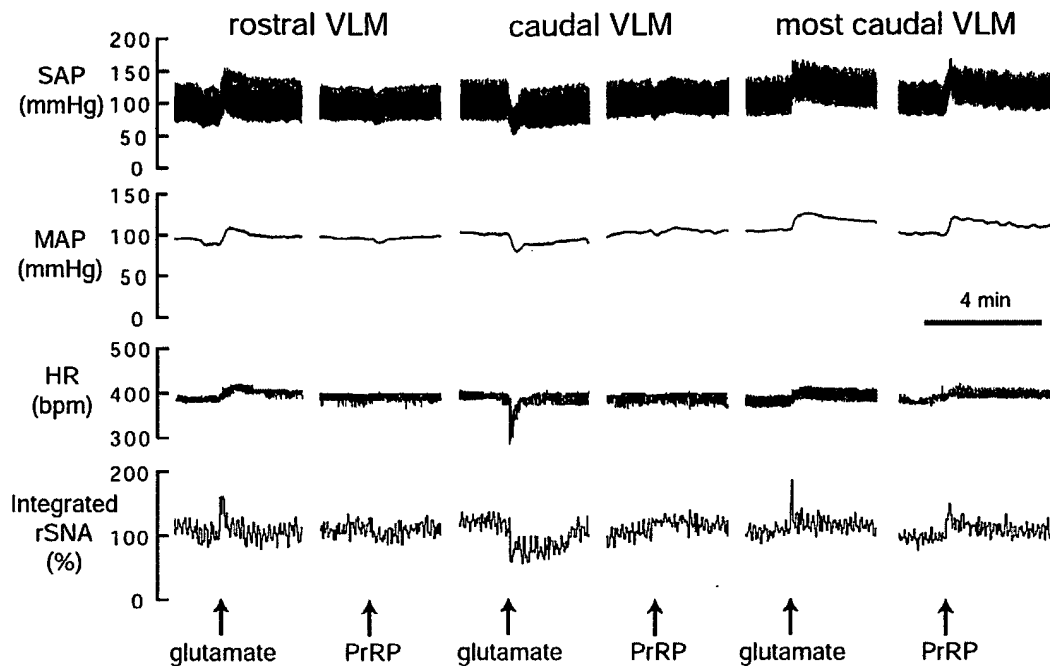


Fig. 2. Typical trace showing the effects of PrRP and glutamate microinjection into different regions of the VLM on systemic arterial pressure (SAP), MAP, HR, and integrated rSNA. Glutamate (2.5 nmol) or PrRP (75 pmol) was microinjected at a volume of 50 nl into three VLM regions, i.e. the pressor area in the rostral VLM, the depressor area in the caudal VLM, and the pressor area in the most caudal VLM, defined as the caudal pressor area (CPA), in urethane-anesthetized rats. rSNA is expressed as a percentage of the pre-injection level.

border between the upper cervical cord and the caudal end of the medulla to the level of the caudal parts of the hypoglossal nucleus and the lateral reticular nucleus (levels 1.0–2.2 mm caudal to the obex). The rostral part of their distribution overlapped considerably with the region in which PrRP microinjection was effective in eliciting pressor and sympathoexcitatory responses. Many strongly immunoreactive cell bodies were localized in the medial and ventrolateral parts of the NTS (Fig. 4C). PrRP-ir nerve fibers, which were easily detected by the P2L-1C antibody, were found in the same regions, or regions at somewhat more rostral levels, as those in which PrRP-ir cell bodies were localized. The most caudal VLM contained PrRP-ir nerve fibers with modest immunoreactivity (Fig. 4E), which were, however, consistently observed in a region (0.8–1.6 mm caudal to the obex) that overlapped well with the region in which PrRP microinjection was effective. Numerous PrRP-ir nerve fibers were seen in the medial and ventrolateral parts of the NTS and less markedly in the commissural part of the NTS (Fig. 4G). In addition to these regions, dense PrRP-ir nerve fibers were observed in the vicinity of the AP and, at more rostral levels, in the dorsomedial part of the NTS (Fig. 4I) and the central part of the intermediate reticular nucleus, in which no PrRP-ir cell bodies were found. Neither PrRP-ir nerve fibers nor cell bodies were found in the regions that corresponded to the depressor area in the caudal VLM or the pressor area in the rostral VLM.

4. Discussion

In the present study we have, for the first time, systematically mapped the caudal part of the VLM in terms of the PrRP-induced changes in MAP, HR, and rSNA. The results clearly demonstrate that PrRP induces pressor and sympathoexcitatory responses only when injected into the most caudal VLM, which has been recognized as the CPA in the rat [4,24,26]. The study has also shown that this PrRP-responsive region overlaps neuroanatomically with a region that contains PrRP-ir neurons.

In the present experiments, a microinjection method was employed to identify the PrRP-responsive sites. The pressor responses to PrRP are not due to an extensive diffusion of the peptide away from the injection site or to a leakage to the cerebrospinal fluid. This is because an equivolume injection of glutamate solution successfully distinguished the well-defined pressor and depressor areas within the caudal VLM and failed to induce any significant response just outside these areas (Fig. 1). This is further supported by the fact that microinjection of angiotensin II (which is a more potent peptide in inducing pressor responses than PrRP when applied to cerebrospinal fluid [29]) did not affect MAP at the same injection site (Table 1). It is unlikely that the responses were induced by non-specific stimuli following the microinjection. This is because the PrRP-induced responses were dose-dependent in spite of a constant injection volume (Fig. 3), while an equivolume

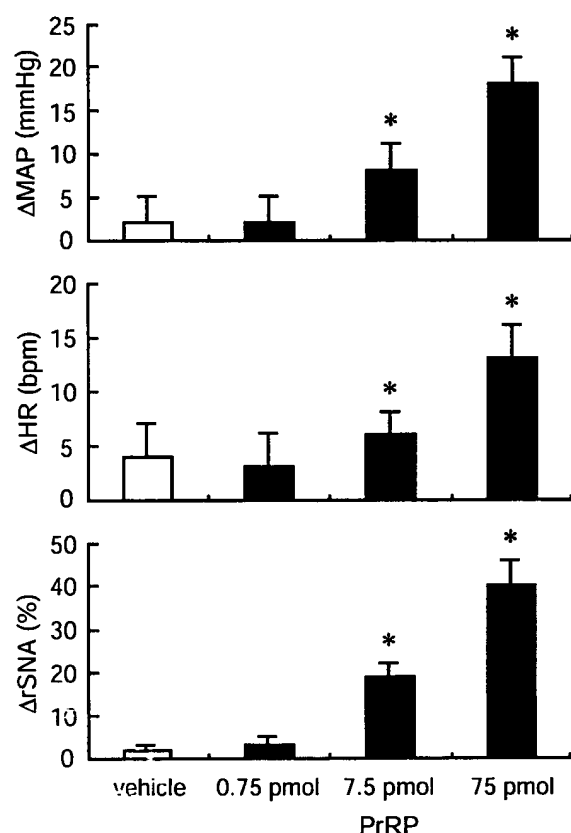


Fig. 3. Dose-dependent increases in MAP, HR, and rSNA evoked by microinjection of PrRP into the most caudal VLM. The vehicle solution or different doses of PrRP (50 nl) were injected, and maximal increases in these parameters after each microinjection were subjected to statistical analysis. rSNA is expressed as a percentage of the pre-injection level. Values are the mean and S.E.M. *Significantly different at $P < 0.05$ from the response produced by vehicle microinjection.

injection of vehicle or the solutions containing other peptides (angiotensin II, TRH, and CRH) with a comparable dose and osmolarity did not produce any detectable changes in MAP and rSNA (Table 1). Therefore, the pressor and sympathoexcitatory responses induced by

PrRP are considered as a specific action of this peptide in the close vicinity of the injection sites.

The PrRP-responsive sites were localized in a highly restricted area within the caudal VLM, which extended from the caudal end of the nucleus ambiguus to a level 0.6–1.0 mm more caudal and was located around the dorsal part of the lateral reticular nucleus (Fig. 1, right). The extent of the PrRP-responsive sites overlapped well with the pressor and sympathoexcitatory area in the most caudal VLM that was delineated by the mapping study with glutamate (Fig. 1, left). This area has been defined as the site where a selective stimulation of cell bodies with neuroexcitatory amino acids produces pressor [4,24,26] and sympathoexcitatory [24] responses; the area is therefore recognized as the CPA. The coincidence between the PrRP-responsive area and the CPA suggests that PrRP activates, directly or indirectly the neurons in the CPA that are responsible for the pressor and sympathoexcitatory responses. Recently, Campos and McAllen [4] have shown that glutamate injection into the CPA causes an increase in the firing rate of sympathetic premotor neurons in the rostral VLM and a subsequent pressor response. Thus, it is possible that the sympathoexcitation elicited by microinjection of PrRP into the CPA is mediated by sympathetic premotor neurons in the rostral VLM. It remains to be investigated whether endogenous PrRP in the CPA is involved in the activation of these sympathoexcitatory CPA neurons.

Samson et al. [29] have reported that, in conscious rats, i.c.v. administration of PrRP evokes a pressor response. The present study has provided evidence that the CPA is a possible action site responsible for the pressor response induced by i.c.v. applied PrRP. Unilateral microinjection of PrRP into the CPA caused an increase in MAP within 3 s after the injection, accompanied by an increase in rSNA. The short onset of the pressor response and the association of sympathoexcitation suggests that the pressor response shown in the present study is primarily due to neurogenic vasoconstriction mediated by the CPA and the core circuit for cardiovascular regulation in the medulla. The results of the present studies do not exclude the possibility, however,

Table 1

The effects of glutamate, angiotensin II (Ang II), corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) microinjected in the pressor area of the most caudal ventrolateral medulla (VLM) on mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (rSNA)

	MAP (mmHg)		HR (bpm)		rSNA (%)	
	Before	After	Before	After	Before	After
Glutamate	93±4	113±3*	373±12	393±16*	100	141±6*
Ang II	94±4	92±6	367±13	368±12	100	100±2
CRH	97±4	96±4	354±7	356±6	100	101±2
TRH	95±3	94±2	355±8	357±7	100	101±1

In four rabbits, 2.5 nmol of glutamate was microinjected into the pressor area in the most caudal VLM. After a recovery period, 50 pmol of either Ang II ($n=4$), TRH ($n=3$), or CRH ($n=3$) was microinjected into the same injection site at intervals of at least 30 min. Pre-injection levels and peak values after the microinjection are indicated as mean±S.E.M. rSNA is expressed as a percentage of the pre-injection level. *Significantly different from the pre-injection level at $P < 0.05$.

Table 2

The effects of glutamate and prolactin-releasing peptide (PrRP) microinjected in the nucleus tractus solitarius (NTS) and the area postrema (AP) on mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (rSNA)

	MAP (mmHg)		HR (bpm)		rSNA (%)	
	Before	After	Before	After	Before	After
Glutamate in NTS	92±2	61±2*	375±11	318±15*	100	15±3*
PrRP in NTS	94±2	93±3	374±11	376±8	100	99±2
Glutamate in AP	94±3	93±2	371±10	372±10	100	101±1
PrRP in AP	91±3	92±4	369±10	364±12	100	101±2

Glutamate (2.5 nmol) or PrRP (75 pmol) was injected at a volume of 50 nl into the NTS and AP ($n=4$). Pre-injection levels and peak values after the microinjection are indicated as mean±S.E.M. rSNA is expressed as a percentage of the pre-injection level. *Significantly different from the pre-injection level at $P<0.05$.

that i.c.v. injected PrRP also acts on other brain regions, e.g. the hypothalamic structures, and activates the neural and/or humoral pathways that affect blood pressure. Indeed, i.c.v. administration of PrRP activates a majority of the CRH-containing neurons in the paraventricular nucleus (PVN) of the hypothalamus and stimulates release of CRH [21], which could cause a pressor response [16]. Furthermore, i.c.v. administration of PrRP increases plasma levels of vasopressin [19], which has a direct vasoconstrictor effect on the blood vessels. Thus, exogenous and endogenous PrRP is likely to affect blood pressure via the multiple responsive sites and pathways including the CPA. However, the contribution, if any, of circulating hormones such as vasopressin, to the pressor response induced by microinjection of PrRP into the CPA is less important, because the pressor response was accompanied by a sympathoexcitation, which consistently overcame any possible baroreflex-mediated sympathoinhibition induced by release of vasoactive hormones.

The medullary cardiovascular regions contain a variety of neuropeptides, such as angiotensin II, neuropeptide Y, and substance P, many of which are involved as neuromodulators in cardiovascular and other autonomic regulations [8,12,27]. It has been supposed that, in the hypothalamus, PrRP may have a neuromodulatory action. This supposition is based on the findings that PrRP and noradrenaline synergistically stimulate the CRH-mediated ACTH release [20], and that the CRH-containing neurons in the PVN are surrounded by PrRP-ir nerve fibers [21], which arise from noradrenaline neurons in the medulla [22]. Thus, it seems likely that, similar to other neuropeptides seen in the medulla, PrRP acts on the neurons in the CPA as a neuromodulator to regulate cardiovascular function. Immunohistochemical and in situ hybridization studies have demonstrated that PrRP-ir cell bodies are localized in the caudal VLM, as well as the NTS [5,18,20,22,28]. This was also confirmed in the present study. On the other hand, the distribution of PrRP-ir neurons in the CPA, which is located more caudally to the main cluster of the A1 cells, has not been clearly documented. Interestingly, the present immunohistochemical results demonstrate that a significant number of the PrRP-ir

cell bodies and modestly dense nerve fibers are distributed in the PrRP-responsive area of the CPA. It is known that the noradrenaline-containing neurons in the caudal VLM exist beyond the caudal end of the nucleus ambiguus to the levels of the pyramidal decussation, and further caudal in the spinal cord [14]. Therefore, it is tempting to speculate that PrRP acts on the PrRP-ir noradrenergic neurons in the CPA, either directly or indirectly. Supporting this possibility, there is evidence that some of the hypothalamic cells that secrete luteinizing hormone-releasing hormone, vasopressin or oxytocin have receptors for their own peptide, through which the release of the peptide is autoregulated [7,15].

We could not induce any detectable changes in MAP, HR, and rSNA by injecting PrRP into the caudal depressor VLM (Figs. 1 and 2), the NTS or the AP (Table 2), in which PrRP and/or its receptors are also seen [5,18,20,22,28]. One possible explanation for the ineffectiveness in these regions is that these cardiovascular regions may not mediate at least the rapid-onset stimulatory action of PrRP on the baseline cardiovascular variables. The effects of PrRP microinjection into these areas on the reflex and/or long-term cardiovascular responses remain to be clarified. However, in conscious rabbits, most of the caudal VLM neurons excited by increases in blood pressure are TH-negative (non-A1), while a majority of the neurons excited by sustained hypotension or hypoxia are TH-positive (A1) [13,17]. Therefore, it is possible that PrRP acts on the TH-positive cells in the depressor area in the caudal VLM and participates in cardiovascular control during sustained hypotension and hypoxia.

In summary, the present results have demonstrated that PrRP selectively induces pressor and sympathoexcitatory responses when applied to the pressor area in the most caudal VLM; but not to the pressor area in the rostral VLM, the depressor area in the caudal VLM or the NTS. The pressor response may be due, at least partly, to its sympathoexcitatory action. This unique nature of PrRP contrasts well with a number of neuropeptides localized in the medullary cardiovascular regions, such as angiotensin II [2,8,30], whose major action sites are located in the more rostral VLM as well as in the NTS and AP. The

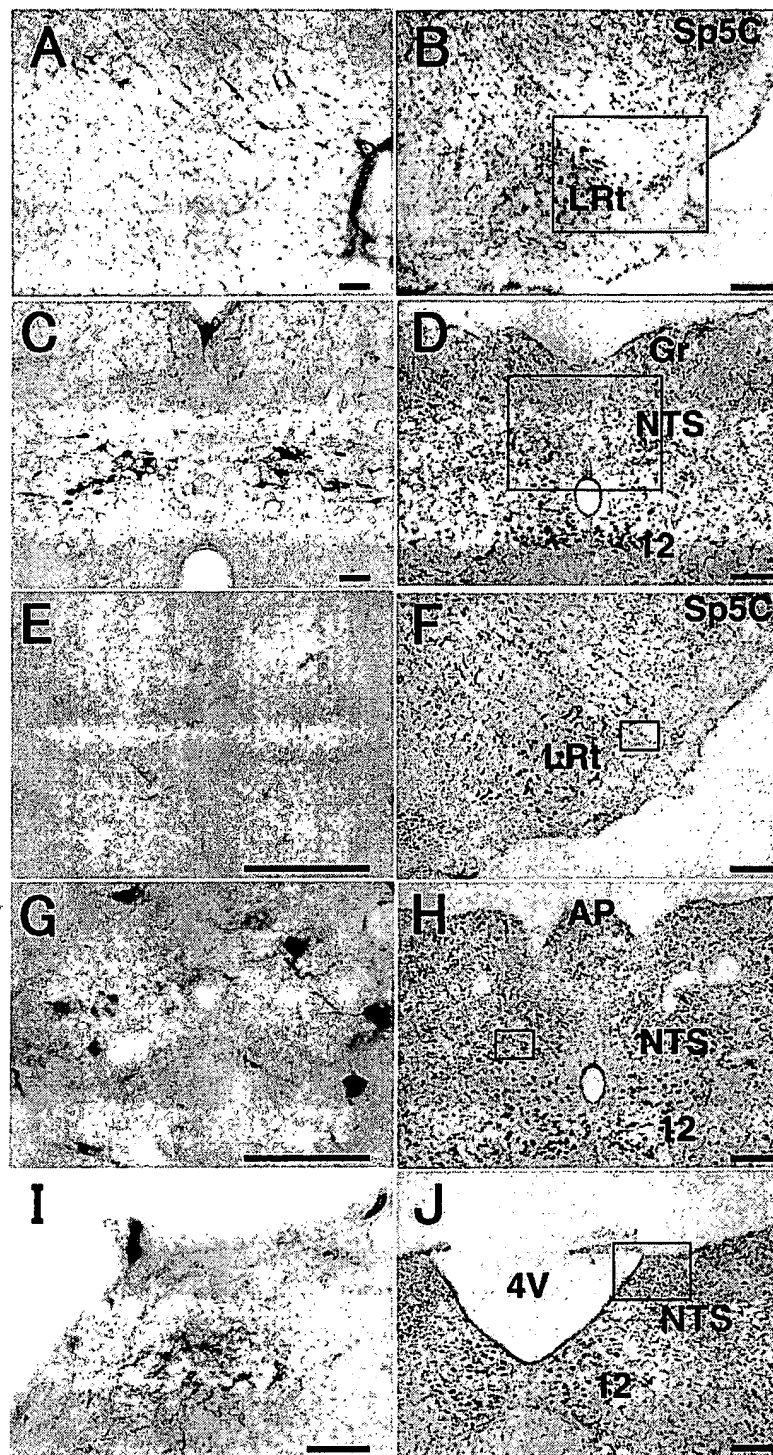


Fig. 4. Photomicrographs showing PrRP-ir cell bodies and nerve fibers in the rat medulla. Left panels are enlarged photomicrographs of the parts of PrRP-immunostained sections that correspond to the parts indicated with rectangles in their right-side panels, and right panels are photomicrographs of the Nissl-stained adjacent sections. (A,C) PrRP-ir cell bodies in the most caudal VLM and the NTS, respectively, stained with the P2L-1T antibody; (E,G,I) PrRP-ir nerve fibers in the most caudal VLM and the medial and the dorsomedial parts of the NTS, respectively, stained with the P2L-1C antibody. Scale bars: 70 μ m in the left panels and 250 μ m in the right panels. 4V, 4th ventricle; 12, hypoglossal nucleus; AP, area postrema; Gr, gracile nucleus; LRT, lateral reticular nucleus; NTS, nucleus tractus solitarius; Sp5C, caudal part of the spinal trigeminal nucleus.

results suggest the existence of sympathoexcitatory neurons activated by PrRP in the most caudal VLM, which may participate in maintenance and/or adaptation of blood pressure control.

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Research report

A novel action of the newly described prolactin-releasing peptides: cardiovascular regulation

Willis K. Samson ^{a,*}, Zachary T. Resch ^b, Tonya C. Murphy ^b^a Department of Pharmacological and Physiological Sciences, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104-1028, USA^b Department of Physiology, University of North Dakota School of Medicine, Grand Forks, ND 58202-9037, USA

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Abstract

The physiological relevance of the recently described prolactin-releasing peptides (PrRPs) has yet to be established. Here, we demonstrate the low potency of the PrRPs (minimum effective dose: 100 nM), compared to that observed for thyrotropin-releasing hormone (TRH, minimum effective dose: 1.0 nM), to stimulate prolactin (PRL) release from cultured pituitary cells harvested from lactating female rats. Anatomic studies question the role of these peptides in neuroendocrine control of lactotroph function. Instead, peptide and peptide receptor mapping studies suggest potential actions in hypothalamus and brainstem unrelated to the control of anterior pituitary hormone secretion. Intracerebroventricular (i.c.v.) administration of both PrRP-20 and PrRP-31 (0.4 and 4.0 nmol) resulted in significantly increased mean arterial blood pressure in conscious, unrestrained rats [peak elevations vs. baseline: PrRP-20, 10% and 16%, low and high dose peptide; PrRP-31, 7% and 10%; compared to the response to 0.1 nmol angiotensin II (A II), 15–17%]. Similar doses of peptide did not significantly alter water drinking in response to overnight fluid deprivation, or thirst or salt appetite in response to an isotonic hypovolemic challenge. Thus, the effect on blood pressure appeared relatively specific. We suggest that these peptides, identified originally as ligands for a receptor found in abundance in pituitary gland, play a broader role in brain function and that the ability of them to stimulate PRL release may not represent their primary biologic function. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Blood pressure; Cardiovascular regulation; Prolactin

1. Introduction

Two novel peptides, identified as ligands that bound the orphan receptor, hGR₃, which is the human counterpart of rat UHR-1 [26] in the pituitary gland, have been reported to possess prolactin-releasing factor (PRF) activity [8,9]. However, we reported that the PRF activity of these prolactin-releasing peptides (PrRPs), PrRP-20 and PrRP-31, which are post-translational modifications of the same gene product, are weak secretagogues when compared to the conventional PRFs, like thyrotropin-releasing hormone (TRH), and are active in vitro only in cells from female pituitary glands [23]. This gender-biased PRF activity and relative lack of potency in vitro was subsequently confirmed in vivo [14] by the demonstration of the requirement of 10-fold higher doses of PrRP-31 to stimulate

prolactin (PRL) release when given i.v. to male as compared to female rats.

Although these peptides were originally named because of the presence of their receptors in anterior pituitary gland and their ability to stimulate PRL release from cells harvested from lactating female rats [8], PrRP receptor and PrRP mRNA have been reported in numerous central nervous system (CNS) sites [7,15]. Immunohistochemical localization studies have identified the presence of PrRP-containing neurons not only in hypothalamus but also in limbic forebrain, including the bed nucleus of the stria terminalis and the amygdala, and in brainstem regions known to be important in central cardiovascular regulation [6,10,12,17,27]. In fact, a role for the PrRPs other than in the neuroendocrine regulation of PRL secretion must be envisioned since very little, if any, peptide immunoreactivity can be visualized in the external layer of the median eminence [10,12,15,17,27]. Instead, PrRP innervation of autonomic centers in hypothalamus [10,12,15,17] and brainstem [6,12,17] and co-localization of PrRP with tyro-

* Corresponding author. Fax: +1-314-577-8554; e-mail: samsonwk@slu.edu

sine hydroxylase expressing medullary A1 and A2 neurons, suggest a broader role for the peptides, perhaps in the CNS regulation of cardiovascular function. We verify here the relatively weak PRF activity of the PrRPs in anterior pituitary cells harvested from lactating female rats; however, we demonstrate for the first time selective CNS actions of the PrRPs not related to neuroendocrine function, instead, to the CNS control of autonomic function.

2. Materials and methods

Rat PrRPs (PrRP-20 and PrRP-31), TRH and angiotensin II (A II) were obtained from Phoenix Pharmaceuticals (Mt. View, CA) and diluted in physiological saline (0.9% NaCl).

2.1. Anterior pituitary cell cultures

Lactating female rats (250–300 g, days 18–21 lactation; Harlan Sprague–Dawley, Indianapolis, IN) were separated from their pups and sacrificed by decapitation as approved by the University Animal Care and Use Committee. Anterior pituitary glands were collected and mechanically dispersed in trypsin [24]. Cells were aliquoted into 24-well plates (ca. 300,000 cells/well) and incubated for 72 h in Medium 199 (pH 7.3) containing 20 mM HEPES, 10% horse serum, and 1% antibiotic/antimycotic (all from Gibco-BRL, Grand Island, NY) in room air at 37°C. On the day of experimentation, cells were washed with fresh test medium [Medium 199, 20 mM HEPES, 1% penicillin–streptomycin (all from Gibco-BRL) and 0.1% BSA and 0.02 nM bacitracin (both from Sigma, St. Louis, MO), pH 7.3, 37°C] and exposed for 1 h to test medium alone or medium containing log molar doses of PrRP-20, PrRP-31 or TRH ranging from 1.0 to 100 nM. Incubations were terminated by removal of medium. PRL content of the incubation medium was determined by radioimmunoassay using the rat kit provided by the National Hormone and Pituitary Program (NHPP, NIDDK) with rPRL-RP3 standard, as previously described [24]. Significant differences ($p < 0.05$) within groups of test peptides were determined by ANOVA and subsequent Newman–Keuls multiple comparison testing.

2.2. Animal preparation for in vivo experimentation

Adult male rats (200–250 g, Sprague–Dawley, Harlan) were housed individually (lights on 0500 h, off 1900 h) following implantation of a chronic indwelling cannula (23-gauge, stainless steel) into the right lateral cerebroventricle under tribromoethanol (2.5% in saline, 1 ml/100 g b.wt., i.e., Aldrich, Rouses Point, NY) with the aid of a stereotaxic device as previously described [21,22]. Animals were employed for experimentation only after return to

presurgery weights (minimally 5 days after surgery). The presence of a dipsogenic response to 0.1 nmol A II administered via the cannula before the initiation of the experiment and at its termination verified cannula placement in the lateral cerebroventricle. All animal surgery and testing (vide infra) procedures were approved by the Institutional Animal Care and Use Committee.

2.3. Blood pressure paradigm

Rats were anesthetized a second time with tribromoethanol, minimally 5 days after implantation of the cerebroventricular cannula, and the left external carotid artery exposed through a surgically prepared incision (1–2 cm). A cannula (PE-50, filled with 0.9% NaCl containing 250 U/ml heparin) was inserted, tunneled s.c. to exit on the dorsum of the neck, and sutured in place [22]. The incision was closed and animals allowed to recover. Minimally 3 h later, the exteriorized cannula was flushed with heparinized saline and attached to a pressure transducer (Digi-Med Blood Pressure Analyzer, Micro-Med, Louisville, KY). Blood pressure was monitored during a 1-h stabilization period. Intracerebroventricular (i.c.v.) injections (2 μ l) were made in conscious, freely moving rats with the aid of the implanted guide cannula and a 25-gauge stainless steel injection cannula attached to a 10- μ l Hamilton syringe (Reno, NV). Saline vehicle or vehicle containing either 0.1 nmol A II or one of the test doses of PrRP (0.4 or 4.0 nmol) was administered over a 20-s interval. All animals tested received three injections during the experimental period: saline vehicle to control for injection artifact, A II as an internal positive response control, and either the low or the high dose of one of the PrRPs. Blood pressure was monitored at 5-s intervals and recorded as means of the pressures present during the last 15 s of a 1-min sampling period. Data were analyzed by paired *t*-test comparing 10-min means before and after injection of the test substance. An outcome with a probability of $p < 0.05$ was considered significant.

2.4. Ingestive behavior paradigms

Additional groups of male rats with implanted lateral cerebroventricular cannulas were employed for the examination of potential behavioral effects of the peptides.

2.4.1. Dehydration-induced water drinking

Individually housed rats were fed lab chow and tap water ad libitum for 5 days and daily water consumption monitored. On the first day of experimentation, both food and water were removed overnight [25]. Eighteen hours later, prior to reinstatement of the water bottle, rats were divided into two groups. One group (time matched controls) received an injection i.c.v. of 2 μ l saline vehicle and the other group received 4.0 nmol PrRP-20 in vehicle i.c.v.

Water bottles were returned to the cages and intakes monitored for 3 h. The experiment was repeated in two more groups of animals: time matched, vehicle injection controls and rats receiving 4.0 nmol PrRP-31 i.c.v. Amounts of water consumed at each time point were compared by unpaired *t*-test between peptide injected rats and their time-matched controls.

2.4.2. Thirst and salt appetite in response to isotonic hypovolemic challenge

After acclimatization of rats to individual cages and the availability of two drinking bottles (one containing tap water and another containing 0.3 M NaCl), animals were subjected to a procedure that establishes an isotonic hypovolemic challenge, and subsequent effects of PrRP-20 and PrRP-31 on water and saline ingestion monitored as previously described [5,20]. This challenge has been shown to uncover physiologically relevant effects of several hypothalamic peptides on ingestive behaviors, in particular, peptides that can also act in CNS to alter blood pressure and cardiovascular function [5,20]. Polyethylene glycol (PEG) was administered (5 ml, 30% w/w, in 0.15 mol/l NaCl, s.c.) on day 1 of testing. After 18 h, during which food and drinking fluids were not present, saline vehicle or vehicle containing 4.0 nmol PrRP-20 or PrRP-31 was injected i.c.v., drinking bottles were returned to the cages, and cumulative intakes of water and saline were monitored for 5 h. Significant differences ($p < 0.05$) between vehicle and peptide injected animals at each sampling interval were determined by *t*-test.

3. Results

3.1. Anterior pituitary cell cultures

In three separate cell harvests from lactating female rats, TRH significantly stimulated PRL release in a dose-

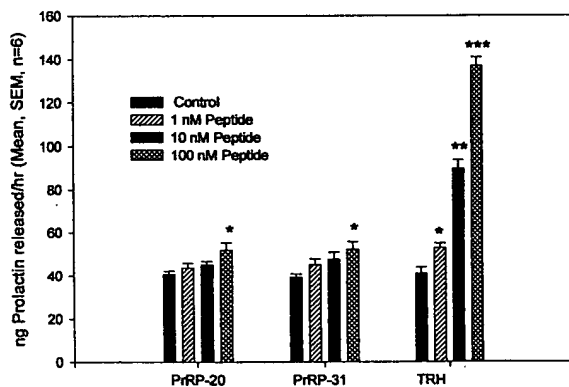


Fig. 1. Comparison of the PRL-releasing activities of the novel PrRPs and TRH, in dispersed anterior pituitary cells harvested from lactating female rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

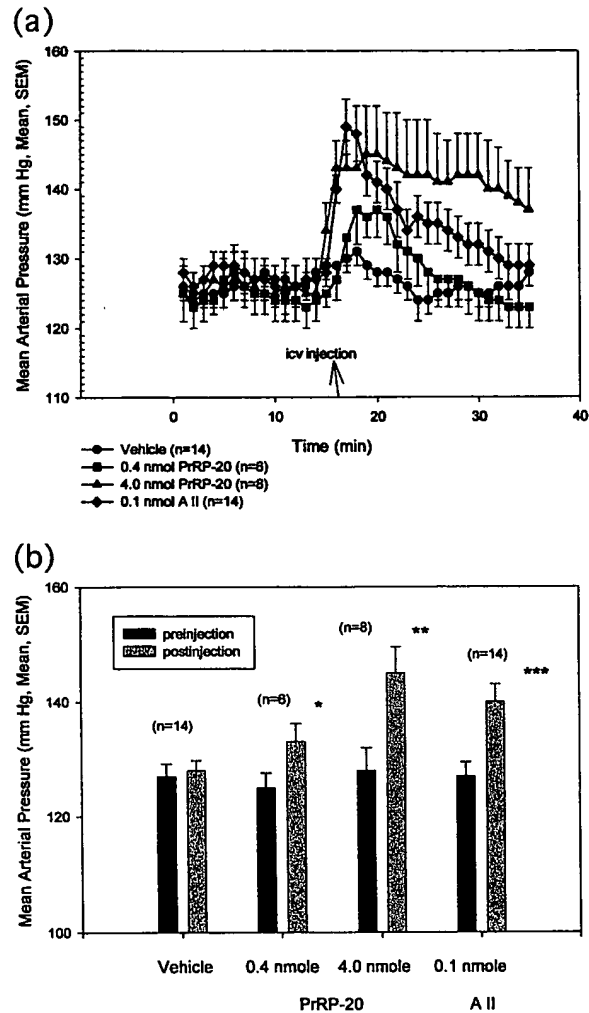


Fig. 2. I.c.v. administration of PrRP (PrRP-20) elevates blood pressure in conscious, unrestrained male rats. Responses are compared to that for a pressor dose of A II. (a) Data are expressed as means for each of 15 min prior to i.c.v. injections (at the 15-min time point) and for each of the next 20 min. (b) Data are expressed as mean pressures observed for the 10-min intervals before and after i.c.v. injections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre- vs. post-injection.

related fashion. The minimum effective PRF dose of TRH was 1.0 nmol. Data from one experiment are presented in Fig. 1. Although PRF activity was observed for both PrRP-20 and PrRP-31, the minimum effective dose for both peptides was 100 nmol. No significant stimulation of PRL release was observed with lower doses of these peptides.

3.2. Blood pressure paradigm

Stable, pre-injection baseline blood pressures were observed in all animals. Saline vehicle injection failed to significantly alter mean arterial pressure (10 min pre-injec-

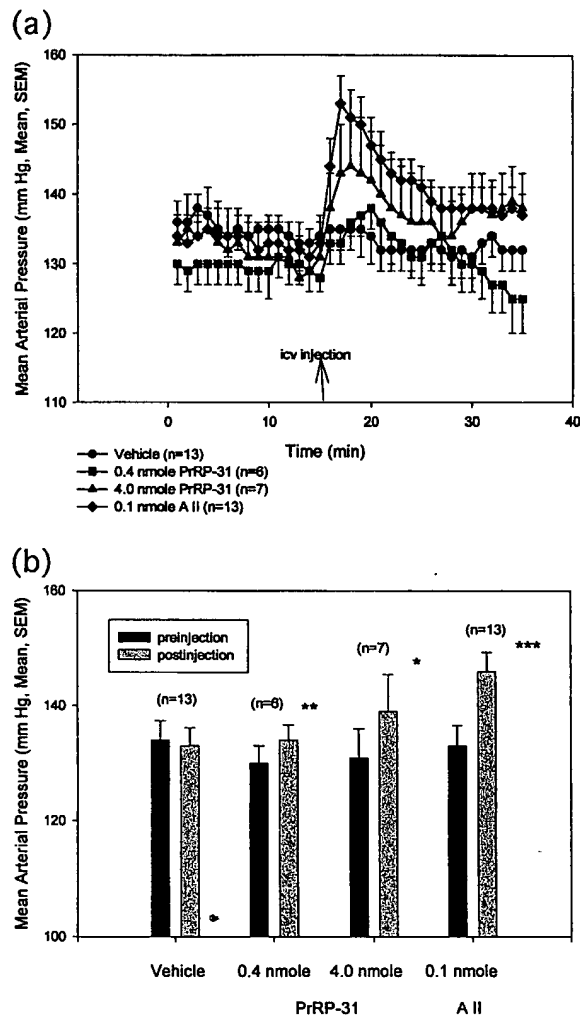


Fig. 3. I.c.v. administration of PrRP (PrRP-31) elevates blood pressure in conscious, unrestrained male rats. Responses are compared to that for a pressor dose of A II. (a) Data are expressed as means for each of 15 min prior to i.c.v. injections (at the 15-min time point) and for each of the next 20 min. (b) Data are expressed as mean pressures observed for the 10-min intervals before and after i.c.v. injections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre- vs. post-injection.

tion means vs. 10 min post-saline vehicle injection means, $p > 0.05$ in all cases). In animals tested for blood pressure

responses to PrRP-20, significant elevations ($p < 0.001$) in mean arterial pressure were observed in response to 0.1 nmol A II (Fig. 2a and b), confirming cannula placement in the ventricle and animal responsiveness. Pressures had returned to pre-injection baseline within 20 min following administration of A II. Significant, dose related elevations in pressure were observed in response to 0.4 ($p < 0.05$) and 4.0 nmol ($p < 0.01$) PrRP-20. Mean blood pressures returned to pre-injection control levels in the group receiving 0.4 nmol PrRP-20 within 15 min following the injection. Pressures remained elevated for at least 20 min in the rats receiving the higher dose of PrRP-20 (Fig. 2b), eventually returning to baseline after 25 min (data not shown).

In animals tested for blood pressure responses to PrRP-31, significant elevations ($p < 0.001$) in mean arterial pressure were observed in response to 0.1 nmol A II (Fig. 3a and b), confirming cannula placement in the ventricle and animal responsiveness. Pressures remained elevated for 20 min following administration of A II, returning to baseline after 25 min (data not shown). Significant, elevations in mean arterial pressure were observed in response to 0.4 ($p < 0.01$) and 4.0 nmol ($p < 0.05$) PrRP-31. Mean blood pressures returned to pre-injection control levels in the group receiving 0.4 nmol PrRP-31 within 15 min following the injection. Pressures remained elevated for at least 20 min in the rats receiving the higher dose of PrRP-31 (Fig. 3b), eventually returning to baseline after 25 min (data not shown). Although the magnitude of the 10-min mean response to the higher dose of PrRP-31 exceeded that in response to the lower dose of peptide (+9 vs. +4 mmHg), no dose-response relationship was observed due to the degree of variability in the high dose group of animals.

3.3. Ingestive behavior paradigms

Water drinking in response to overnight water restriction was not significantly affected by treatment with 4.0 nmol PrRP-20 or 4.0 nmol PrRP-31 (Table 1), doses of peptide which when injected similarly into the lateral cerebroventricle resulted in significantly elevated mean arterial pressures.

Similarly, neither thirst (water consumption) nor saline drinking salt appetite) in response to isotonic hypovolemic

Table 1
I.c.v. administration of PrRPs failed to significantly alter water drinking in response to overnight water deprivation

Treatment	Cumulative water intakes (ml, mean \pm S.E.M.)						
	15 min	30 min	45 min	60 min	90 min	120 min	180 min
Group I (n = 5)							
Vehicle control	5.6 \pm 0.8	7.2 \pm 0.7	8.6 \pm 1.2	8.8 \pm 1.3	9.2 \pm 1.5	9.2 \pm 1.5	9.2 \pm 1.5
4.0 nmol PrRP-20	5.4 \pm 0.8	7.4 \pm 1.0	7.6 \pm 1.5	9.2 \pm 1.5	10.8 \pm 2.1	11.4 \pm 2.6	11.6 \pm 2.8
Group II (n = 7)							
Vehicle control	6.0 \pm 1.2	7.9 \pm 1.6	10.4 \pm 1.2	12.9 \pm 1.1	14.7 \pm 0.8	16.3 \pm 1.0	17.6 \pm 1.4
4.0 nmol PrRP-31	6.1 \pm 0.7	8.9 \pm 1.0	10.3 \pm 1.4	11.7 \pm 1.1	13.1 \pm 1.2	14.6 \pm 1.4	14.9 \pm 1.5

Table 2

I.c.v. administration of PrRPs failed to significantly alter thirst or salt appetite in rats following isotonic hypovolemic challenge

Thirst treatment	Cumulative water intakes (ml, mean \pm S.E.M.)				
	60 min	120 min	180 min	240 min	300 min
<i>Group I (n = 5)</i>					
Vehicle control	7.0 \pm 1.2	10.2 \pm 1.7	11.8 \pm 1.8	14.6 \pm 3.2	15.0 \pm 3.0
4.0 nmol PrRP-20	8.8 \pm 1.4	11.2 \pm 1.2	13.0 \pm 1.4	15.6 \pm 2.1	15.6 \pm 2.1
<i>Group II (n = 6)</i>					
Vehicle control	11.8 \pm 2.6	16.5 \pm 2.7	20.3 \pm 2.4	22.0 \pm 3.3	24.2 \pm 2.9
4.0 nmol PrRP-31	11.7 \pm 1.8	14.8 \pm 1.2	18.0 \pm 2.4	19.3 \pm 2.4	20.5 \pm 2.7
Salt appetite treatment	Cumulative saline intakes (ml, mean \pm S.E.M.)				
	60 min	120 min	180 min	240 min	300 min
<i>Group I (n = 5)</i>					
Vehicle control	4.8 \pm 2.3	7.8 \pm 3.3	10.0 \pm 4.2	12.0 \pm 5.4	12.0 \pm 5.4
4.0 nmol PrRP-20	3.2 \pm 1.1	6.4 \pm 1.7	9.2 \pm 2.4	9.8 \pm 2.8	9.8 \pm 2.8
<i>Group II (n = 6)</i>					
Vehicle control	2.3 \pm 1.0	4.0 \pm 2.2	5.5 \pm 2.2	7.3 \pm 2.7	7.7 \pm 2.8
4.0 nmol PrRP-31	2.8 \pm 0.9	5.3 \pm 2.1	5.8 \pm 2.4	6.7 \pm 2.4	7.5 \pm 2.5

challenge were significantly affected by treatment with 4.0 nmol PrRP-20 or 4.0 nmol PrRP-31 (Table 2).

4. Discussion

It had long been recognized that the major hypothalamic influence over PRL release from the anterior pituitary gland was inhibitory in nature, expressed by dopamine released from tuberoinfundibular neurons that projected to the external layer of the median eminence [11]. However, abundant evidence suggested the presence of other hypothalamic factors that may, under certain circumstances, regulate hormone secretion [1,2,11,19,24]. Numerous peptides with PRL-releasing activity have been described; however, their activity is largely dictated by ambient levels of dopamine. Only TRH, of all the peptides demonstrated to possess PRF activity, is generally accepted to be a physiologic regulator of PRL secretion, and yet controversy still exists. Evidence from studies employing antibodies and antagonists has accumulated for a physiologic role for vasoactive intestinal peptide [1] and oxytocin [19]. Several labs have attempted for some time to identify novel peptides with physiologically relevant PRF-like activity [2].

The announcement of the bioactivity of the PrRPs promised continued interest in the field of neuroendocrinology. However, the bioactivity of the PrRPs was not as robust as that of TRH and indeed a gender-biased activity was reported, since cells harvested from female animals were more responsive than similar cells from males [23]. This observation has been extended recently by *in vivo* studies from the laboratory of the discoverers of

the peptides who demonstrated that, to elicit PRL release, higher doses of PrRP were needed when administered *i.v.* into males than into females [14]. Also, we report here that the bioactivity of the PrRPs in cells harvested from lactating female rats is similarly not as potent as that of TRH. The reason for the discrepancy between our results in cells from lactating animals and the level of potency described by Hinuma et al. [8] is unlikely due to the peptide preparation employed. Our findings with the Phoenix Pharmaceuticals peptide (Fig. 1) were mirrored in companion studies (data not shown), and indeed in some of the same cell harvests, using peptide obtained from the Peptide Institute (Osaka, Japan). These findings, when coupled with the developing literature that reported the absence of peptide in the external layer of the median eminence [12,17,27], therefore, predicting low levels in the hypophyseal portal plasma, questioned the physiological relevance of the observed PRF activities. It is possible that the diffuse expression of PrRP receptor mRNA in anterior pituitary, that is not restricted to any one cell type [17], indicates a non-neuroendocrine, perhaps paracrine action of locally produced peptide [12], unrelated to the unique control of lactotroph function. In the absence of neutralizing antibodies, antagonists, or gene overexpression/deletion models, the question of physiologic relevance cannot yet be satisfactorily answered. In the meantime, the apparent mismatch of receptor message and peptide message/localization [7] has stimulated a re-examination of the biologic role(s) played by these two novel peptides.

We have been studying the coincident pituitary and brain actions of numerous neuropeptides for some time [18] and speculated that the presence of PrRP receptors in brain sites unrelated to neuroendocrine function [7,8,17] and PrRP immunoreactivity in areas known to be impor-

tant in the control of cardiovascular function [6,7,12–15,17,27] predicted behavioral or autonomic actions of the peptides in vivo. We focused on two well-characterized ingestive behaviors due to the localization of PrRP peptide in sites known to influence thirst and salt appetite [5]. The presence of PrRP immunoreactivity in brainstem and particularly in A1 and A2 noradrenergic neurons of the caudal medulla [6,17] led us to examine possible in vivo actions of the PrRPs on cardiovascular function.

Both peptides, when injected into the lateral cerebroventricle stimulated a robust increase in mean arterial blood pressure in conscious, freely moving rats. We can only speculate on the potential sites of actions of the peptides to stimulate an elevation in pressure and indeed, can only hypothesize at this point that the responses observed were due to increases in sympathetic outflow from the medulla. Experiments such as those we have previously reported for the adrenomedullin gene-derived peptides [22] and the natriuretic peptides [16] will have to be conducted to verify the possible activation of sympathetic tone by the PrRPs, as will direct electrophysiologic recordings [3,4]. Similarly, site-specific infusions of the peptides must now be conducted in an attempt to identify more precisely the localization of the actions of the PrRPs in brain. Already, peptide and receptor distribution studies suggest actions in lateral reticular nucleus and nucleus ambiguus in the caudal medulla, as well as in the nucleus of the solitary tract [6,17]. More rostrally, site-specific actions may occur in the paraventricular nucleus of the hypothalamus, the bed nucleus of the stria terminalis and the amygdala. Additionally, the hierarchy of the effect of the PrRPs needs to be studied in order to determine if these peptides, like proadrenomedullin in N-terminal 20 peptide [22], recruit other neuropeptides to express their cardiovascular effects in brain. The specificity of the blood pressure elevating actions is attested to, at least in part, the failure of effective doses of PrRPs to alter thirst in two independent models of water drinking and to affect salt appetite as well. Here, a unique dissociation of actions is observed, since other neuropeptides, which similarly alter cardiovascular function when administered i.c.v., also significantly alter thirst and salt appetite [18].

Does this mean that the PrRPs are not important regulators of PRL secretion? No, it does not. Instead, our data offer an alternative role for these peptides in brain function than merely the neuroendocrine regulation of lactotroph function, and suggest that investigators designing in vivo experiments to study the neuroendocrine actions of the peptides should recognize the potential for additional confounding effects, which may alter, by themselves, the response of the animal to a variety of stimuli. These data also identify yet another family of peptides, the RFamide peptides [9], that may need to be examined in a variety of experimental settings before the hierarchy of neuronal networks controlling basal and reflexive cardiovascular function can be determined.

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Research report

A novel action of the newly described prolactin-releasing peptides: cardiovascular regulation

Willis K. Samson ^{a,*}, Zachary T. Resch ^b, Tonya C. Murphy ^b^a *Department of Pharmacological and Physiological Sciences, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104-1028, USA*^b *Department of Physiology, University of North Dakota School of Medicine, Grand Forks, ND 58202-9037, USA*

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Abstract

The physiological relevance of the recently described prolactin-releasing peptides (PrRPs) has yet to be established. Here, we demonstrate the low potency of the PrRPs (minimum effective dose: 100 nM), compared to that observed for thyrotropin-releasing hormone (TRH, minimum effective dose: 1.0 nM), to stimulate prolactin (PRL) release from cultured pituitary cells harvested from lactating female rats. Anatomic studies question the role of these peptides in neuroendocrine control of lactotroph function. Instead, peptide and peptide receptor mapping studies suggest potential actions in hypothalamus and brainstem unrelated to the control of anterior pituitary hormone secretion. Intracerebroventricular (i.c.v.) administration of both PrRP-20 and PrRP-31 (0.4 and 4.0 nmol) resulted in significantly increased mean arterial blood pressure in conscious, unrestrained rats [peak elevations vs. baseline: PrRP-20, 10% and 16%, low and high dose peptide; PrRP-31, 7% and 10%; compared to the response to 0.1 nmol angiotensin II (A II), 15–17%]. Similar doses of peptide did not significantly alter water drinking in response to overnight fluid deprivation, or thirst or salt appetite in response to an isotonic hypovolemic challenge. Thus, the effect on blood pressure appeared relatively specific. We suggest that these peptides, identified originally as ligands for a receptor found in abundance in pituitary gland, play a broader role in brain function and that the ability of them to stimulate PRL release may not represent their primary biologic function. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Blood pressure; Cardiovascular regulation; Prolactin

1. Introduction

Two novel peptides, identified as ligands that bound the orphan receptor, hGR₃, which is the human counterpart of rat UHR-1 [26] in the pituitary gland, have been reported to possess prolactin-releasing factor (PRF) activity [8,9]. However, we reported that the PRF activity of these prolactin-releasing peptides (PrRPs), PrRP-20 and PrRP-31, which are post-translational modifications of the same gene product, are weak secretagogues when compared to the conventional PRFs, like thyrotropin-releasing hormone (TRH), and are active in vitro only in cells from female pituitary glands [23]. This gender-biased PRF activity and relative lack of potency in vitro was subsequently confirmed in vivo [14] by the demonstration of the requirement of 10-fold higher doses of PrRP-31 to stimulate

prolactin (PRL) release when given i.v. to male as compared to female rats.

Although these peptides were originally named because of the presence of their receptors in anterior pituitary gland and their ability to stimulate PRL release from cells harvested from lactating female rats [8], PrRP receptor and PrRP mRNA have been reported in numerous central nervous system (CNS) sites [7,15]. Immunohistochemical localization studies have identified the presence of PrRP-containing neurons not only in hypothalamus but also in limbic forebrain, including the bed nucleus of the stria terminalis and the amygdala, and in brainstem regions known to be important in central cardiovascular regulation [6,10,12,17,27]. In fact, a role for the PrRPs other than in the neuroendocrine regulation of PRL secretion must be envisioned since very little, if any, peptide immunoreactivity can be visualized in the external layer of the median eminence [10,12,15,17,27]. Instead, PrRP innervation of autonomic centers in hypothalamus [10,12,15,17] and brainstem [6,12,17] and co-localization of PrRP with tyro-

* Corresponding author. Fax: +1-314-577-8554; e-mail: samsonwk@slu.edu

sine hydroxylase expressing medullary A1 and A2 neurons, suggest a broader role for the peptides, perhaps in the CNS regulation of cardiovascular function. We verify here the relatively weak PRF activity of the PrRPs in anterior pituitary cells harvested from lactating female rats; however, we demonstrate for the first time selective CNS actions of the PrRPs not related to neuroendocrine function, instead, to the CNS control of autonomic function.

2. Materials and methods

Rat PrRPs (PrRP-20 and PrRP-31), TRH and angiotensin II (A II) were obtained from Phoenix Pharmaceuticals (Mt. View, CA) and diluted in physiological saline (0.9% NaCl).

2.1. Anterior pituitary cell cultures

Lactating female rats (250–300 g, days 18–21 lactation; Harlan Sprague–Dawley, Indianapolis, IN) were separated from their pups and sacrificed by decapitation as approved by the University Animal Care and Use Committee. Anterior pituitary glands were collected and mechanically dispersed in trypsin [24]. Cells were aliquoted into 24-well plates (ca. 300,000 cells/well) and incubated for 72 h in Medium 199 (pH 7.3) containing 20 mM HEPES, 10% horse serum, and 1% antibiotic/antimycotic (all from Gibco-BRL, Grand Island, NY) in room air at 37°C. On the day of experimentation, cells were washed with fresh test medium [Medium 199, 20 mM HEPES, 1% penicillin–streptomycin (all from Gibco-BRL) and 0.1% BSA and 0.02 nM bacitracin (both from Sigma, St. Louis, MO), pH 7.3, 37°C] and exposed for 1 h to test medium alone or medium containing log molar doses of PrRP-20, PrRP-31 or TRH ranging from 1.0 to 100 nM. Incubations were terminated by removal of medium. PRL content of the incubation medium was determined by radioimmunoassay using the rat kit provided by the National Hormone and Pituitary Program (NHPP, NIDDK) with rPRL-RP3 standard, as previously described [24]. Significant differences ($p < 0.05$) within groups of test peptides were determined by ANOVA and subsequent Newman–Keuls multiple comparison testing.

2.2. Animal preparation for *in vivo* experimentation

Adult male rats (200–250 g, Sprague–Dawley, Harlan) were housed individually (lights on 0500 h, off 1900 h) following implantation of a chronic indwelling cannula (23-gauge, stainless steel) into the right lateral cerebroventricle under tribromoethanol (2.5% in saline, 1 ml/100 g b.wt., i.e., Aldrich, Rouses Point, NY) with the aid of a stereotaxic device as previously described [21,22]. Animals were employed for experimentation only after return to

presurgery weights (minimally 5 days after surgery). The presence of a dipsogenic response to 0.1 nmol A II administered via the cannula before the initiation of the experiment and at its termination verified cannula placement in the lateral cerebroventricle. All animal surgery and testing (*vide infra*) procedures were approved by the Institutional Animal Care and Use Committee.

2.3. Blood pressure paradigm

Rats were anesthetized a second time with tribromoethanol, minimally 5 days after implantation of the cerebroventricular cannula, and the left external carotid artery exposed through a surgically prepared incision (1–2 cm). A cannula (PE-50, filled with 0.9% NaCl containing 250 U/ml heparin) was inserted, tunneled s.c. to exit on the dorsum of the neck, and sutured in place [22]. The incision was closed and animals allowed to recover. Minimally 3 h later, the exteriorized cannula was flushed with heparinized saline and attached to a pressure transducer (Digi-Med Blood Pressure Analyzer, Micro-Med, Louisville, KY). Blood pressure was monitored during a 1-h stabilization period. Intracerebroventricular (i.c.v.) injections (2 μ l) were made in conscious, freely moving rats with the aid of the implanted guide cannula and a 25-gauge stainless steel injection cannula attached to a 10- μ l Hamilton syringe (Reno, NV). Saline vehicle or vehicle containing either 0.1 nmol A II or one of the test doses of PrRP (0.4 or 4.0 nmol) was administered over a 20-s interval. All animals tested received three injections during the experimental period: saline vehicle to control for injection artifact, A II as an internal positive response control, and either the low or the high dose of one of the PrRPs. Blood pressure was monitored at 5-s intervals and recorded as means of the pressures present during the last 15 s of a 1-min sampling period. Data were analyzed by paired *t*-test comparing 10-min means before and after injection of the test substance. An outcome with a probability of $p < 0.05$ was considered significant.

2.4. Ingestive behavior paradigms

Additional groups of male rats with implanted lateral cerebroventricular cannulas were employed for the examination of potential behavioral effects of the peptides.

2.4.1. Dehydration-induced water drinking

Individually housed rats were fed lab chow and tap water *ad libitum* for 5 days and daily water consumption monitored. On the first day of experimentation, both food and water were removed overnight [25]. Eighteen hours later, prior to reinstatement of the water bottle, rats were divided into two groups. One group (time matched controls) received an injection i.c.v. of 2 μ l saline vehicle and the other group received 4.0 nmol PrRP-20 in vehicle i.c.v.

Water bottles were returned to the cages and intakes monitored for 3 h. The experiment was repeated in two more groups of animals: time matched, vehicle injection controls and rats receiving 4.0 nmol PrRP-31 i.c.v. Amounts of water consumed at each time point were compared by unpaired *t*-test between peptide injected rats and their time-matched controls.

2.4.2. Thirst and salt appetite in response to isotonic hypovolemic challenge

After acclimatization of rats to individual cages and the availability of two drinking bottles (one containing tap water and another containing 0.3 M NaCl), animals were subjected to a procedure that establishes an isotonic hypovolemic challenge, and subsequent effects of PrRP-20 and PrRP-31 on water and saline ingestion monitored as previously described [5,20]. This challenge has been shown to uncover physiologically relevant effects of several hypothalamic peptides on ingestive behaviors, in particular, peptides that can also act in CNS to alter blood pressure and cardiovascular function [5,20]. Polyethylene glycol (PEG) was administered (5 ml, 30% w/w, in 0.15 mol/l NaCl, s.c.) on day 1 of testing. After 18 h, during which food and drinking fluids were not present, saline vehicle or vehicle containing 4.0 nmol PrRP-20 or PrRP-31 was injected i.c.v., drinking bottles were returned to the cages, and cumulative intakes of water and saline were monitored for 5 h. Significant differences ($p < 0.05$) between vehicle and peptide injected animals at each sampling interval were determined by *t*-test.

3. Results

3.1. Anterior pituitary cell cultures

In three separate cell harvests from lactating female rats, TRH significantly stimulated PRL release in a dose-

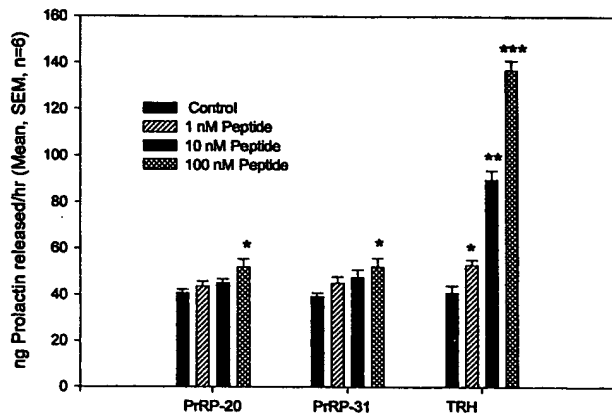


Fig. 1. Comparison of the PRL-releasing activities of the novel PrRPs and TRH, in dispersed anterior pituitary cells harvested from lactating female rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

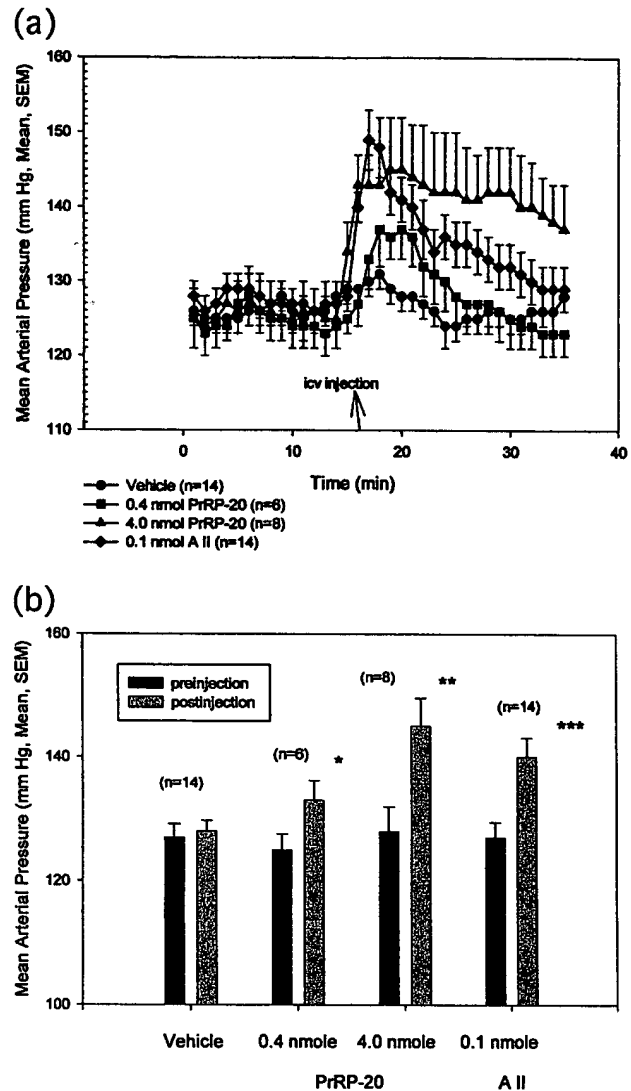


Fig. 2. I.c.v. administration of PrRP (PrRP-20) elevates blood pressure in conscious, unrestrained male rats. Responses are compared to that for a pressor dose of A II. (a) Data are expressed as means for each of 15 min prior to i.c.v. injections (at the 15-min time point) and for each of the next 20 min. (b) Data are expressed as mean pressures observed for the 10-min intervals before and after i.c.v. injections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre- vs. post-injection.

related fashion. The minimum effective PRF dose of TRH was 1.0 nmol. Data from one experiment are presented in Fig. 1. Although PRF activity was observed for both PrRP-20 and PrRP-31, the minimum effective dose for both peptides was 100 nmol. No significant stimulation of PRL release was observed with lower doses of these peptides.

3.2. Blood pressure paradigm

Stable, pre-injection baseline blood pressures were observed in all animals. Saline vehicle injection failed to significantly alter mean arterial pressure (10 min pre-injec-

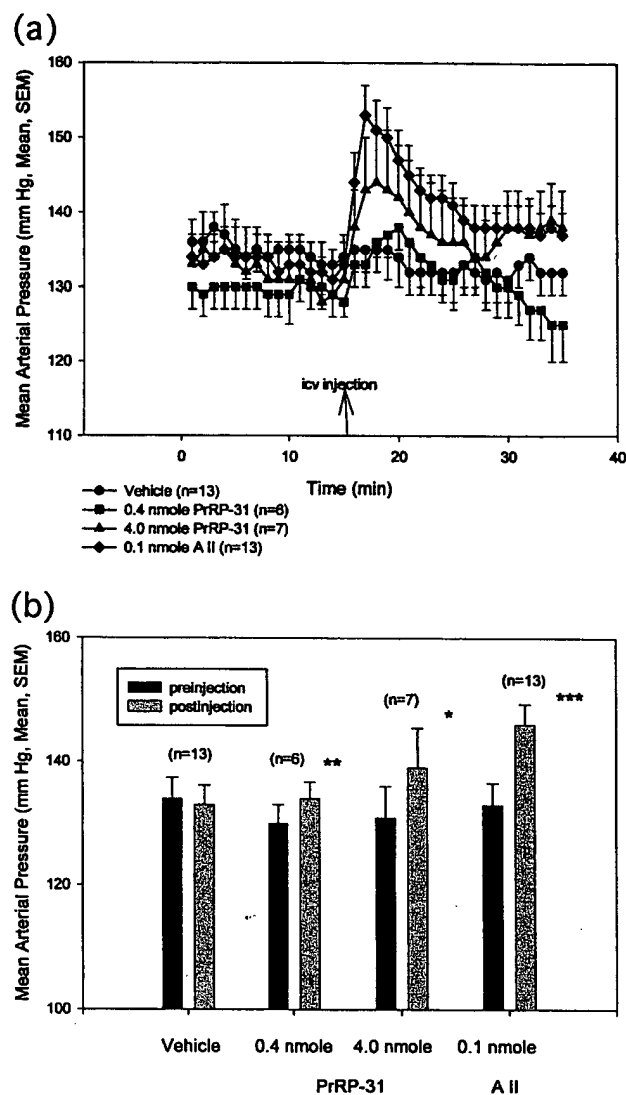


Fig. 3. I.c.v. administration of PrRP (PrRP-31) elevates blood pressure in conscious, unrestrained male rats. Responses are compared to that for a pressor dose of A II. (a) Data are expressed as means for each of 15 min prior to i.c.v. injections (at the 15-min time point) and for each of the next 20 min. (b) Data are expressed as mean pressures observed for the 10-min intervals before and after i.c.v. injections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre- vs. post-injection.

tion means vs. 10 min post-saline vehicle injection means, $p > 0.05$ in all cases). In animals tested for blood pressure

responses to PrRP-20, significant elevations ($p < 0.001$) in mean arterial pressure were observed in response to 0.1 nmol A II (Fig. 2a and b), confirming cannula placement in the ventricle and animal responsiveness. Pressures had returned to pre-injection baseline within 20 min following administration of A II. Significant, dose related elevations in pressure were observed in response to 0.4 ($p < 0.05$) and 4.0 nmol ($p < 0.01$) PrRP-20. Mean blood pressures returned to pre-injection control levels in the group receiving 0.4 nmol PrRP-20 within 15 min following the injection. Pressures remained elevated for at least 20 min in the rats receiving the higher dose of PrRP-20 (Fig. 2b), eventually returning to baseline after 25 min (data not shown).

In animals tested for blood pressure responses to PrRP-31, significant elevations ($p < 0.001$) in mean arterial pressure were observed in response to 0.1 nmol A II (Fig. 3a and b), confirming cannula placement in the ventricle and animal responsiveness. Pressures remained elevated for 20 min following administration of A II, returning to baseline after 25 min (data not shown). Significant, elevations in mean arterial pressure were observed in response to 0.4 ($p < 0.01$) and 4.0 nmol ($p < 0.05$) PrRP-31. Mean blood pressures returned to pre-injection control levels in the group receiving 0.4 nmol PrRP-31 within 15 min following the injection. Pressures remained elevated for at least 20 min in the rats receiving the higher dose of PrRP-31 (Fig. 3b), eventually returning to baseline after 25 min (data not shown). Although the magnitude of the 10-min mean response to the higher dose of PrRP-31 exceeded that in response to the lower dose of peptide (+9 vs. +4 mmHg), no dose-response relationship was observed due to the degree of variability in the high dose group of animals.

3.3. Ingestive behavior paradigms

Water drinking in response to overnight water restriction was not significantly affected by treatment with 4.0 nmol PrRP-20 or 4.0 nmol PrRP-31 (Table 1), doses of peptide which when injected similarly into the lateral cerebroventricle resulted in significantly elevated mean arterial pressures.

Similarly, neither thirst (water consumption) nor saline drinking salt appetite) in response to isotonic hypovolemic

Table 1

I.c.v. administration of PrRPs failed to significantly alter water drinking in response to overnight water deprivation

Treatment	Cumulative water intakes (ml, mean \pm S.E.M.)						
	15 min	30 min	45 min	60 min	90 min	120 min	180 min
Group I (n = 5)							
Vehicle control	5.6 \pm 0.8	7.2 \pm 0.7	8.6 \pm 1.2	8.8 \pm 1.3	9.2 \pm 1.5	9.2 \pm 1.5	9.2 \pm 1.5
4.0 nmol PrRP-20	5.4 \pm 0.8	7.4 \pm 1.0	7.6 \pm 1.5	9.2 \pm 1.5	10.8 \pm 2.1	11.4 \pm 2.6	11.6 \pm 2.8
Group II (n = 7)							
Vehicle control	6.0 \pm 1.2	7.9 \pm 1.6	10.4 \pm 1.2	12.9 \pm 1.1	14.7 \pm 0.8	16.3 \pm 1.0	17.6 \pm 1.4
4.0 nmol PrRP-31	6.1 \pm 0.7	8.9 \pm 1.0	10.3 \pm 1.4	11.7 \pm 1.1	13.1 \pm 1.2	14.6 \pm 1.4	14.9 \pm 1.5

Table 2

I.c.v. administration of PrRPs failed to significantly alter thirst or salt appetite in rats following isotonic hypovolemic challenge

Thirst treatment	Cumulative water intakes (ml, mean \pm S.E.M.)				
	60 min	120 min	180 min	240 min	300 min
<i>Group I (n = 5)</i>					
Vehicle control	7.0 \pm 1.2	10.2 \pm 1.7	11.8 \pm 1.8	14.6 \pm 3.2	15.0 \pm 3.0
4.0 nmol PrRP-20	8.8 \pm 1.4	11.2 \pm 1.2	13.0 \pm 1.4	15.6 \pm 2.1	15.6 \pm 2.1
<i>Group II (n = 6)</i>					
Vehicle control	11.8 \pm 2.6	16.5 \pm 2.7	20.3 \pm 2.4	22.0 \pm 3.3	24.2 \pm 2.9
4.0 nmol PrRP-31	11.7 \pm 1.8	14.8 \pm 1.2	18.0 \pm 2.4	19.3 \pm 2.4	20.5 \pm 2.7
Salt appetite treatment	Cumulative saline intakes (ml, mean \pm S.E.M.)				
	60 min	120 min	180 min	240 min	300 min
<i>Group I (n = 5)</i>					
Vehicle control	4.8 \pm 2.3	7.8 \pm 3.3	10.0 \pm 4.2	12.0 \pm 5.4	12.0 \pm 5.4
4.0 nmol PrRP-20	3.2 \pm 1.1	6.4 \pm 1.7	9.2 \pm 2.4	9.8 \pm 2.8	9.8 \pm 2.8
<i>Group II (n = 6)</i>					
Vehicle control	2.3 \pm 1.0	4.0 \pm 2.2	5.5 \pm 2.2	7.3 \pm 2.7	7.7 \pm 2.8
4.0 nmol PrRP-31	2.8 \pm 0.9	5.3 \pm 2.1	5.8 \pm 2.4	6.7 \pm 2.4	7.5 \pm 2.5

challenge were significantly affected by treatment with 4.0 nmol PrRP-20 or 4.0 nmol PrRP-31 (Table 2).

4. Discussion

It had long been recognized that the major hypothalamic influence over PRL release from the anterior pituitary gland was inhibitory in nature, expressed by dopamine released from tuberoinfundibular neurons that projected to the external layer of the median eminence [11]. However, abundant evidence suggested the presence of other hypothalamic factors that may, under certain circumstances, regulate hormone secretion [1,2,11,19,24]. Numerous peptides with PRL-releasing activity have been described; however, their activity is largely dictated by ambient levels of dopamine. Only TRH, of all the peptides demonstrated to possess PRF activity, is generally accepted to be a physiologic regulator of PRL secretion, and yet controversy still exists. Evidence from studies employing antibodies and antagonists has accumulated for a physiologic role for vasoactive intestinal peptide [1] and oxytocin [19]. Several labs have attempted for some time to identify novel peptides with physiologically relevant PRF-like activity [2].

The announcement of the bioactivity of the PrRPs promised continued interest in the field of neuroendocrinology. However, the bioactivity of the PrRPs was not as robust as that of TRH and indeed a gender-biased activity was reported, since cells harvested from female animals were more responsive than similar cells from males [23]. This observation has been extended recently by in vivo studies from the laboratory of the discoverers of

the peptides who demonstrated that, to elicit PRL release, higher doses of PrRP were needed when administered i.v. into males than into females [14]. Also, we report here that the bioactivity of the PrRPs in cells harvested from lactating female rats is similarly not as potent as that of TRH. The reason for the discrepancy between our results in cells from lactating animals and the level of potency described by Hinuma et al. [8] is unlikely due to the peptide preparation employed. Our findings with the Phoenix Pharmaceuticals peptide (Fig. 1) were mirrored in companion studies (data not shown), and indeed in some of the same cell harvests, using peptide obtained from the Peptide Institute (Osaka, Japan). These findings, when coupled with the developing literature that reported the absence of peptide in the external layer of the median eminence [12,17,27], therefore, predicting low levels in the hypophyseal portal plasma, questioned the physiological relevance of the observed PRF activities. It is possible that the diffuse expression of PrRP receptor mRNA in anterior pituitary, that is not restricted to any one cell type [17], indicates a non-neuroendocrine, perhaps paracrine action of locally produced peptide [12], unrelated to the unique control of lactotroph function. In the absence of neutralizing antibodies, antagonists, or gene overexpression/deletion models, the question of physiologic relevance cannot yet be satisfactorily answered. In the meantime, the apparent mismatch of receptor message and peptide message/localization [7] has stimulated a re-examination of the biologic role(s) played by these two novel peptides.

We have been studying the coincident pituitary and brain actions of numerous neuropeptides for some time [18] and speculated that the presence of PrRP receptors in brain sites unrelated to neuroendocrine function [7,8,17] and PrRP immunoreactivity in areas known to be impor-

tant in the control of cardiovascular function [6,7,12–15,17,27] predicted behavioral or autonomic actions of the peptides in vivo. We focused on two well-characterized ingestive behaviors due to the localization of PrRP peptide in sites known to influence thirst and salt appetite [5]. The presence of PrRP immunoreactivity in brainstem and particularly in A1 and A2 noradrenergic neurons of the caudal medulla [6,17] led us to examine possible in vivo actions of the PrRPs on cardiovascular function.

Both peptides, when injected into the lateral cerebroventricle stimulated a robust increase in mean arterial blood pressure in conscious, freely moving rats. We can only speculate on the potential sites of actions of the peptides to stimulate an elevation in pressure and indeed, can only hypothesize at this point that the responses observed were due to increases in sympathetic outflow from the medulla. Experiments such as those we have previously reported for the adrenomedullin gene-derived peptides [22] and the natriuretic peptides [16] will have to be conducted to verify the possible activation of sympathetic tone by the PrRPs, as will direct electrophysiologic recordings [3,4]. Similarly, site-specific infusions of the peptides must now be conducted in an attempt to identify more precisely the localization of the actions of the PrRPs in brain. Already, peptide and receptor distribution studies suggest actions in lateral reticular nucleus and nucleus ambiguus in the caudal medulla, as well as in the nucleus of the solitary tract [6,17]. More rostrally, site-specific actions may occur in the paraventricular nucleus of the hypothalamus, the bed nucleus of the stria terminalis and the amygdala. Additionally, the hierarchy of the effect of the PrRPs needs to be studied in order to determine if these peptides, like proadrenomedullin in N-terminal 20 peptide [22], recruit other neuropeptides to express their cardiovascular effects in brain. The specificity of the blood pressure elevating actions is attested to, at least in part, the failure of effective doses of PrRPs to alter thirst in two independent models of water drinking and to affect salt appetite as well. Here, a unique dissociation of actions is observed, since other neuropeptides, which similarly alter cardiovascular function when administered i.c.v., also significantly alter thirst and salt appetite [18].

Does this mean that the PrRPs are not important regulators of PRL secretion? No, it does not. Instead, our data offer an alternative role for these peptides in brain function than merely the neuroendocrine regulation of lactotroph function, and suggest that investigators designing in vivo experiments to study the neuroendocrine actions of the peptides should recognize the potential for additional confounding effects, which may alter, by themselves, the response of the animal to a variety of stimuli. These data also identify yet another family of peptides, the RFamide peptides [9], that may need to be examined in a variety of experimental settings before the hierarchy of neuronal networks controlling basal and reflexive cardiovascular function can be determined.

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